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The kit Ligand, Stem Cell Factor

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I. Introduction

Spontaneous mutations affecting inbred mice often provide an opportunity to understand much about the functions of gene products long before those products have been identified. This clearly was true in the case of stem cell factor (SCF) and the stem cell factor receptor (SCFR), the product of the c-*kit* gene. Thus, much of what is now understood about the importance of SCF and the SCFR in hematopoiesis and other complex developmental programs affecting melanocytes, germ cells, and mast cells was predicted based on an analysis of the phenotype of mice with mutations affecting SCF or its receptor.

Indeed, it has been known for decades that mice with a double dose of mutations at either the dominant white spotting (*W*) locus on chromosome 5 or the steel (*Sl*) locus on chromosome 10 exhibit a hypoplastic, macrocytic anemia, sterility and a lack of cutaneous melanocytes (reviewed in Russell, 1979; Silvers, 1979a). Transplantation and embryo fusion studies employing *W* or *Sl* mutant and congenic normal mice, or *in vitro* analyses employing cells or tissues derived from these animals, indicated that the deficits in the *W* mutant mice are expressed by the cells in the affected lineages, whereas those in the *Sl* mutant animals are expressed by microenvironmental cells necessary for the normal development of the affected lineages (Russell, 1979; Silvers, 1979a). The complementary nature of the phenotypic abnormalities expressed by *W* or *Sl* mutant mice suggested that the *W* locus might encode a receptor expressed by hematopoietic cells, melanocytes, and germ cells, whereas the *Sl* locus might encode the corresponding ligand (Russell, 1979).

Kitamura *et al.* (1978) and Kitamura and Go (1979) reported that mutations at *W* or *Sl* also profoundly affect mast cell development. They demonstrated that the virtual absence of mast cells in W/W^v mice, like the anemia of these animals, reflected an abnormality intrinsic to the affected lineage (Kitamura *et al.*, 1978) whereas the mast cell deficiency of Sl/Sl^d mice, which could not be corrected by bone mar-

row transplantation from the congenic normal (+/+) mice, reflected an abnormality in the microenvironments necessary for normal mast cell development (Kitamura and Go, 1979). Kitamura's finding that transplantation of bone marrow cells from the congenic +/+ mice or from beige (C57BL/6-*bg*/*bg*) mice [whose mast cells can be identified unequivocally because of their giant cytoplasmic granules (Chi and Lagunoff, 1975)] repaired the mast cell deficiency of the W/W^v mice provided clear evidence that mast cells were derived from precursors that reside in the bone marrow. This work also showed that mutations at *W* had a more profound effect on the mast cell than on any other hematopoietic lineage. Although this observation at first was of interest primarily to those working on the mast cell, it is now clear that this cell type has properties that are especially well suited for structural and functional analyses of the *W* and *Sl* gene products.

Recent work has confirmed Russell's (1979) hypothesis that *W* encodes a receptor, the tyrosine kinase receptor encoded by the c-*kit* gene (Chabot *et al.*, 1988; Geissler *et al.*, 1988a,b), and that *Sl* encodes the corresponding ligand, which has been named (in alphabetical order) kit ligand (KL) (Huang *et al.*, 1990), mast cell growth factor (MGF) (Williams *et al.*, 1990; Copeland *et al.*, 1990; Anderson *et al.*, 1990), steel factor (SLF or SF) (Witte, 1990; D. E. Williams *et al.*, 1992), and stem cell factor (Zsebo *et al.*, 1990a,b; Martin *et al.*, 1990). This review uses the terms SCF for the ligand and SCFR for the receptor.

By tranducing extracellular signals transmitted by their cognate ligands, receptor tyrosine kinases importantly regulate cell survival, proliferation, and differentiation (Ullrich and Schlessinger, 1990). As predicted based on the phenotypic abnormalities expressed by *W* or *Sl* mutant mice, SCF has been shown to promote hematopoiesis and mast cell development, as well as melanocyte survival and proliferation, and to influence the survival and proliferation of primordial germ cells. Other findings, such as the expression of high levels of the SCFR or SCF in the central nervous system, or the expression of the SCFR on lymphocytes, had not been expected, because *W* or *Sl* mutant mice were not known to exhibit central nervous system (CNS) abnormalities and, in general, these mutants have normal numbers of lymphocytes and normal immune function. Finally, work has shown that SCF can directly promote secretion of mast cell mediators and also can augment mast cell mediator release in response to activation through the cells' FcεRI. These observations were the first, but probably will not be the last, to indicate that SCF can regulate the secretory function of cells that express the SCFR, as well as influence their development.

II. The Stem Cell Factor Receptor, Encoded by the c-*kit* Gene

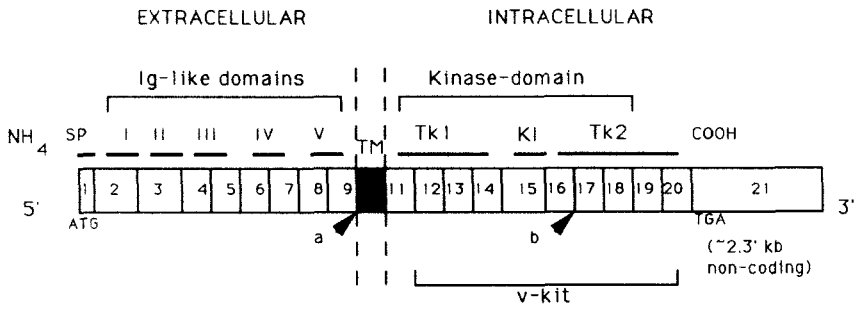
A. v-*kit* AND c-*kit*

The *kit* gene was first characterized as the viral oncogene (v-*kit*) of a feline sarcoma virus (HZ4-FeSV) (Besmer *et al.*, 1986). cDNA cloning of the corresponding cellular sequences from both mouse and human tissues confirmed that v-*kit* arose by transduction and truncation of a cellular gene—the protooncogene c-*kit* (Yarden *et al.*, 1987; Qiu *et al.*, 1988). The murine c-*kit* gene was first cloned from a mouse brain cDNA library (Qiu *et al.*, 1988). The largest cDNA isolated encoded 5.1 of the full-length 5.5-kilobase (kb) pair mRNAs detected in the brain by Northern analysis. Besmer and associates (1986; Qiu *et al.*, 1988) and Yarden *et al.* (1987) noted that the predicted amino acid sequences of v-*kit* and c-*kit*, respectively, displayed extensive homology with the tyrosine kinase receptor superfamily, particularly with members of what is now considered the family of type III transmembrane receptor kinases (RTK), such as the transmembrane receptors for colony-stimulating factor-1 (CSF-1R, encoded by c-*fms*) and the receptors for platelet-derived growth factor (PDGFR-α and -β). The extracellular domain of the SCFR (Fig. 1) includes a signal peptide and a 500-amino acid (aa) ligand-binding domain with five immunoglobulin (Ig)-like regions that contain several intramolecular disulfide bonds and potential N-glycosylation sites (Yarden *et al.*, 1987; Qiu *et al.*, 1988). A 23-amino acid hydrophobic domain anchors the receptor in the plasma membrane. The cytoplasmic portion comprises a 433-aa intracellular segment that contains the kinase region (residues 575–915), an ATP-binding site (residue 622), an autophosphorylation site (residue 821), and a poorly conserved, noncatalytic, hydrophilic stretch of 77 amino acids, known as the kinase insert (KI) domain, that divides the kinase domain in half (Yarden *et al.*, 1987; Qiu *et al.*, 1988). The KI domains of the RTK type III receptors are believed to be involved in the binding of SH2 domains of cytoplasmic signal transduction proteins [see Section IV and Ullrich and Schlessinger (1990)].

The RTK type III family also includes a subgroup whose members have longer extracellular regions, with seven rather than five Ig-like domains. These include a recently discovered family of receptors that includes two receptors for vascular permeability/vascular endothelial growth factor (VPF) (Shibuya *et al.*, 1990; Matthews *et al.*, 1991a,b; Rosnet *et al.*, 1991, 1993; Terman *et al.*, 1991, 1992a,b; Aprelikova *et al.*, 1992; de Vries *et al.*, 1992).

In both mice and humans, c-*kit* can undergo tissue-specific alterna-

A.



B.

Intron Number	Location (human cDNA bp)	Intron Size(kb) Mouse	Human
1	88	16	> 30
2	358	1.9	2.6
3	640	1.8	1.2
4	777	1.8	4.0
5	946	5.2	3.5
6	1136	1.9	2.3
7	1252	>9	8.0
8	1367	0.8	2.3
9	1561	1.5	1.3
10	1668	0.1	0.1
11	1795	0.3	0.3
12	1900	0.1	0.1
13	2011	12	1.2
14	2162	1.5	2.0
15	2254	0.8	0.5
16	2382	1.5	1.1
17	2505	>8	3.3
18	2617	0.1	0.1
19	2717	0.5	0.4
20	2823	1.2	1.1

FIG. 1. Structure of the c-*kit* gene and the SCFR. (A) The structure of the c-*kit* cDNA is shown with the exons boxed and numbered and the location of the ATG translation start and TGA translation stop codons indicated below. The locations of important regions of the SCFR are indicated by bars above the exon(s) that encode them. These include the signal peptide (SP), the five immunoglobulin-like domains (I–V) in the extracellular ligand-binding domain, the transmembrane (TM) region, the two cytoplasmic regions (Tk1 and Tk2), which together comprise the kinase domain and the intervening kinase insertion (KI) region. (a) The use of alternative 5' splice donor sites at the 3' end of exon nine gives rise to the alternatively spliced c-*kit* transcripts that have been described in mouse and human cell lines or tissues (Yarden *et al.*, 1987; Qiu *et al.*, 1988; Reith *et al.*, 1991; Gokkel *et al.*, 1992; Giebel *et al.*, 1992; Vandenbark *et al.* 1992). (b) The use of an alternative promoter in intron 16 gives rise to the truncated 3.2-kb c-*kit* transcript in mouse spermatids, as described by Rossi *et al.* (1992). The location of the cDNA region comprising the virally transduced v-*kit* oncogene is indicated below the

tive splicing (Reith *et al.*, 1990; Vandenbark *et al.*, 1992; Geibel *et al.*, 1992) (Fig. 1). Thus *in vitro*-derived mast cells and midgestation placenta express two c-*kit* transcripts (plus or minus an in-frame insertion, codons 510–513) encoding Gly–Asn–Asn–Lys in the extracellular domain (Yarden *et al.*, 1987; Reith *et al.*, 1991). The homologous alternative c-*kit* transcripts (plus or minus codons 510–513) also have been detected in human cells (Yarden *et al.*, 1987; Giebel *et al.*, 1992). The transcript lacking codons 510–513 constitutes the major form of c-*kit* mRNA present in mouse mast cells, bone marrow cells, and fetal liver cells (Reith *et al.*, 1991) as well as in human HEL erythroid/megakaryocytic cells and melanocytes (Giebel *et al.*, 1992).

By Scatchard analysis, both of the SCFR isoforms exhibit similar affinity for soluble SCF and both respond to soluble ligand with similar levels of autophosphorylation and enhanced association with phosphatidylinositol 3′-kinase (P13′K) and phospholipase C-γ1 (PLC-γ1) (Reith *et al.*, 1991; D. E. Williams *et al.*, 1992). However, Reith *et al.* (1991) found that the shorter form of the SCFR, but not the longer form with the four-amino acid insertion, exhibited a low level of apparently constitutive receptor autophosphorylation and association with P13′K and PLC-γ1. The biological significance of the presence of alternative forms of the SCFR is not yet clear, and the tissue distribution of the two known forms of the receptor has not yet been fully defined. However, it has been suggested that low levels of constitutive SCFR activation may be sufficient to promote cell survival, and that, in some cell types, conditions of SCF deprivation may favor expression of the shorter from of the SCFR (Reith *et al.*, 1991; D. E. Williams *et al.*, 1992).

In the mouse, additional variant c-*kit* transcripts are expressed during gametogenesis. Northern analyses have shown that although spermatogonia express the full-length 5.5-kb c-*kit* transcript, in spermatids

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exons. (B) The conservation of the intron/exon structure of mouse and human c-*kit* genes, showing the location of the introns in the human c-*kit* cDNA nucleotide sequence numbered according to Yarden *et al.* (1987). The major c-*kit* TIS employed in several mouse cell lineages maps upstream from the 5′ end of previously published mouse and human cDNAs, so that in the mouse exon 1 is actually 125 bp in length (Yasuda *et al.*, 1993) (see Fig. 6). The location of the TIS in human c-*kit* has not been reported, so the size of the human exon 1 is not known. Comparison of the data of Gokkel *et al.* (1992), Giebel *et al.* (1992), Vandenbark *et al.* (1992), and André *et al.* (1992) shows that the locations of the introns in the mouse and human c-*kit* genes are conserved. The sizes of the human introns shown on the right are the averages of determinations of three published reports that are in close agreement (Giebel *et al.*, 1992; Vandenbark *et al.*, 1992; André *et al.*, 1992). The reported size (12 kb) of mouse intron 13 (Gokkel *et al.*, 1992) may have been a typographical error.

the 5.5-kb message is replaced by two shorter transcripts, which are 3.2 and 2.3 kb in length (Sorrentino *et al.*, 1991). The longer of the two spermatid-specific alternative transcripts was cloned and shown to comprise a novel truncated c-*kit* transcript that initiates within an intron immediately upstream from the exon encoding the second part of the split kinase domain. Thus this alternative transcript lacks the coding sequences for the extracellular and transmembrane domains and part of the kinase domain (Sorrentino *et al.*, 1991; Rossi *et al.*, 1992). The truncated protein product potentially encoded by this transcript, which has yet to be identified, would lack the ligand-binding and transmembrane domains as well as the ATP-binding site. Therefore, the biological role of the truncated protein would necessarily be ligand and kinase independent. Rossi *et al.* (1992) proposed that this molecule could provide a matrix for protein–protein interactions, analogous to the function that has been suggested for the *Drosophila* c-*abl* tyrosine kinase.

Using an antibody recognizing an epitope in the kinase domain of the SCF receptor, Matsuda *et al.* (1993) identified strong immunoreactivity in the nucleus of both normal and malignant medullary cells of the human adrenal gland. The presence of SCFR protein in the cell nucleus could reflect the presence of additional alternatively spliced c-*kit* products that encode signals that target the protein to the nucleus. It is also possible that the SCFR is transported to the nucleus by association with distinct proteins, which could include alternative forms of the SCFR ligand SCF. For example, some isoforms of PDGF-α have been found in the cell nucleus (Rakowicz-Szulazynska *et al.*, 1986). Whatever the mechanism involved, this finding, if confirmed, would comprise the first example for the nuclear targeting of transmembrane tyrosine kinase receptors and would suggest that the SCFR may have additional important functions that have yet to be identified.

B. Demonstration of Allelism between *W* and c-*kit*

In 1988, Chabot *et al.* reported that *W* and c-*kit* are very closely linked on mouse chromosome 5; both Chabot *et al.* (1988) and Geissler *et al.* (1988a,b) showed that c-*kit* is included in a small chromosome 5 deletion (W^{19H}) that had been shown to include *W* as well as several other closely linked loci. Geissler *et al.* (1988b) also provided the first direct demonstration that *W* mutations influence the structure and expression of the gene encoding the c-*kit* receptor. These data included the finding that two spontaneous, phenotypically different *W* mutations were associated with distinct rearrangements of the c-*kit*

locus. For one mutation, W^{44}, the site of the genetic rearrangement was shown to be confined to the c-*kit* structural gene, reducing the amount of c-*kit* message but apparently not altering its size. W^{44} most likely represents an insertion within an intervening sequence in the 5′ end of the gene (Geissler *et al.*, 1988b). Regardless of the precise mechanism that accounted for the altered expression of c-*kit* in the tissues of the W^{44}/W^{44} mouse, these results established that a genetic rearrangement confined to the c-*kit* structural gene was sufficient to produce a mutant *W* phenotype and, therefore, strongly supported the hypothesis that the c-*kit* protooncogene is allelic with the dominant-spotting (*W*) locus. Subsequently, additional spontaneous *W* mutations were shown to be associated with alterations in the c-*kit* structural gene, including several which comprise single base pair mutations in the region encoding the kinase domain (Bernstein *et al.*, 1990; Nocka *et al.*, 1990b; Reith *et al.*, 1990; Tan *et al.*, 1990; Larue *et al.*, 1992; Tsujimura *et al.*, 1993).

The demonstration of allelism between *W* and c-*kit* represented a significant advance in efforts to understand the mechanism of *W* gene action. Moreover, mutations at *W* represented the first examples of germ-line mutations in a mammalian protooncogene. As a result, analysis of *W* gene action also will help to clarify the role of protooncogenes in normal mammalian development (Besmer, 1991; A. Bernstein *et al.*, 1991; Forrester *et al.*, 1992).

C. Genomic Organization and Chromosomal Location of c-*KIT*

In the human genome, the c-*kit* locus maps in the centromeric region of chromosome 4, between 4q11 and 4q21 (Yarden *et al.*, 1987; Qiu *et al.*, 1988). This region has a well-defined genetic homology to the region of mouse chromosome 5, which includes the *W* locus (Fig. 2) (Yarden *et al.*, 1987). Several groups have described the intron/exon structure of mouse (Gokkel *et al.*, 1992) and human c-*kit* genes (Vandenbark *et al.*, 1992; Giebel *et al.*, 1992; André *et al.*, 1992). The overall structure of the gene, the sizes of the homologous exons, and the locations of the introns and the sequences at the intron/exon boundaries are highly conserved between humans and mice (Fig. 1). In both species, the gene spans over 80 kb of genomic DNA, and the coding sequence is distributed over 21 exons that range in size from 100 to 200 base pairs.

Exon 1 encodes the 5′ untranslated region of the transcript, the ATG translation initiation codon, and the signal peptide. Exons 2–9 encode the rest of the extracellular ligand-binding domain, exon 10 comprises the hydrophobic transmembrane domain, and exons 11–20 encode the

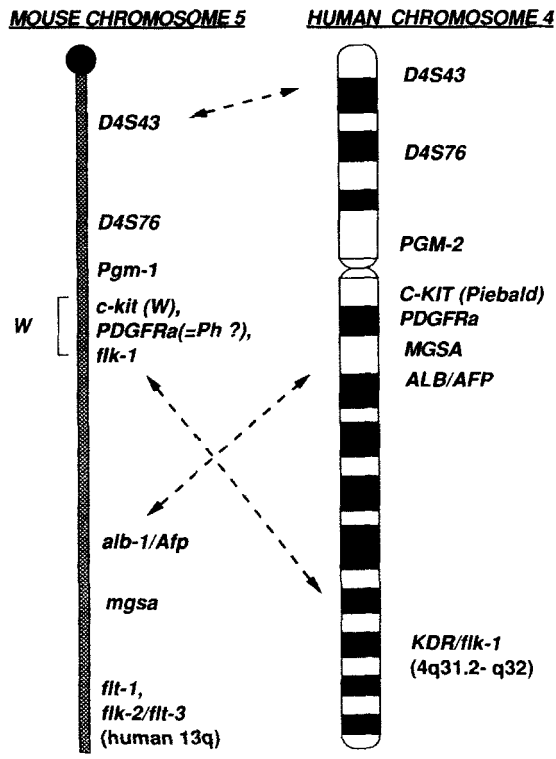

FIG. 2. Chromosomal location of c-*kit* in mouse and human genomes. A partial linkage map of mouse chromosome 5 is shown on the left, as compiled by Hillyard *et al.* (1992), and the cytogenic map of human chromosome 4 is shown on the right (Murray and VanOmmen, 1991). The locations of genes or loci defined by anonymous DNA markers that define the region of homology between these linkage groups are shown to the right of each chromosome. In the mouse, c-*kit* is allelic with *W*; the tandemly linked *PDGFR-α* locus may be allelic with a distinct coat color gene, *Patch (Ph)* (Stephenson *et al.*, 1991; Smith *et al.*, 1991). In man, c-*kit* has been shown to be allelic with the piebald trait (Spritz, 1992; Giebel and Spritz, 1991; Fleischman *et al.*, 1991). The mouse gene *flk-1* encodes a related type III RTK, but with seven rather than five Ig-like domains in the extracellular domain (Matthews *et al.*, 1991a), which was recently shown to be the high-affinity VPFR (Millauer *et al.*, 1993). *flk*-1 maps very near c-*kit* on chromosome 5 (Matthews *et al.*, 1991a). However, the human homologue, *KDR* (Terman *et al.*, 1991), maps far from c-*kit*, on the distal end of human chromosome 4 (Terman *et al.*, 1992a). *flk*-2/*flt*-3, which encodes an RTK with five Ig-like domains in the extracellular domain, was cloned independently by two groups and named *flk-2* (Matthews *et al.*, 1991b) or *flt*-3 (Rosnet *et al.*, 1991). The *flk*-2/*flt*-3 locus maps on the distal end of mouse chromosome 5 and on the long arm of human chromosome 13 (Rosnet *et al.*, 1991). The *flt*-1 gene encodes an RTK that has seven Ig-like domains (Shibuya *et al.*, 1990) and that functions as a second VPFR (de Vries *et al.*, 1992) and is physically linked with *flk*-2/*flt*-3 on both mouse chromosome 5 and human chromosome 13 (Rosnet *et al.*, 1991,1993).

intracellular kinase domain of the protein. The ATP-binding region in the kinase domain is located in exon 12. The kinase insert segment is contained within exons 14 and 15. The translation stop codon and the entire 2.3 kb of 3′ noncoding sequences and the polyadenylation site are derived from exon 21. All of the 5′ donor splice sites and all but one of the 3′ acceptor sites (in intron 4) are highly conserved between the species. The use of alternative splice donor sites at the 5′ end of intervening sequence nine gives rise to the two major c-*kit* transcripts in mouse and human tissues that have been described previously (Vandenbark *et al.*, 1992; Giebel *et al.*, 1992).

Until recently, the c-*kit* transcription initiation site (TIS) and promoter had not been identified in either mouse or human species. Therefore, the exact number of exons in the gene and the correct length of exon 1 were not known. As part of our analysis of the mouse c-*kit* promoter (discussed in Section VI), we have mapped the location of the major c-*kit* TIS 40 base pairs upstream from the 5′ end of the published cDNA. These data demonstrate that exon 1 is 136 base pairs in length and encodes 125 base pairs of 5′ untranslated sequence.

D. Evolutionary Relationship between c-*kit*, c-*fms*, and PDGFR Genes

c-*kit*, c-*fms*, *PDGFR-α* and *PDGFR-β* comprise a group of genes with highly related structures. In addition, c-*kit* and *PDGFR-α* are less than 650 kb apart on mouse chromosome 5 (Qiu *et al.*, 1988; Hsieh *et al.*, 1991; Smith *et al.*, 1991; Stephenson *et al.*, 1991), and are less than 150 kb apart on human chromosome 4q11–q13 (Yarden *et al.*, 1987; Gronwald *et al.*, 1990; Hsieh *et al.*, 1991; Vandenbark *et al.*, 1992; Giebel *et al.*, 1992). c-*fms* and *PDGFR-β* are also tandemly linked on a different pair of mouse and human chromosomes (Roberts *et al.*, 1988), indicating that both of the RTK pairs probably arose from a common ancestral gene by gene duplication events and translocation (Gronwald *et al.*, 1990).

The genomic organization of c-*kit* and c-*fms* is strikingly similar; both have 21 exons and very similar exon/intron boundaries (André *et al.*, 1992; Vandenbark *et al.*, 1992). The nucleotide sequences, exon sizes, and the distribution of the exon/intron boundaries are most highly conserved in the TK1 and TK2 regions of the kinase domains of these genes, reflecting selection for conservation of kinase function. The conservation of RTK gene structure is less striking in the kinase insert regions and in the extracellular domains, reflecting divergence toward receptor-specific properties, such as association with intracellular substrates and cognate ligands, respectively (Rottapel *et al.*,

1991). The *PDGFR-α* and *-β* genes have not been as well studied, but appear to have structures that are generally similar to those of c-*kit* and c-*fms* (Vu *et al.*, 1989; Williams, 1989).

III. The kit Ligand, Stem Cell Factor

A. Identification of the kit Ligand, SCF, as the *Sl* Locus Product

Once allelism between the *W* locus of the mouse and c-*kit* had been established, it did not require a daring conceptual leap to imagine that the kit ligand might be encoded at *Sl*. This hypothesis was in accord with Russell's (1979) proposal that *W* and *Sl* encoded a receptor and its ligand. And even before allelism between *W* and c-*kit* had been identified, several lines of evidence indicated that a receptor–ligand interaction may be important not only in bone marrow stromal cell–hematopoietic cell interactions (Russell, 1979), but also in mast cell–fibroblast interactions (reviewed in Kitamura, 1989; Galli, 1990). Using combinations of mast cells or their precursors and fibroblasts derived from *W* or *Sl* mutant mice, both Kitamura and Fujita (1989; Kitamura *et al.*, 1989; Fujita *et al.*, 1989) and Jarboe and Huff (1989) provided strong biological evidence that fibroblasts can produce cell-associated (Kitamura *et al.*, 1989; Fujita *et al.*, 1989; Kitamura and Fujita, 1989) and soluble (Jarboe and Huff, 1989) forms of the kit ligand. Nocka *et al.* (1990b) then purified and characterized a mouse fibroblast-derived soluble factor, which they designated KL, that stimulated the proliferation of normal mast cells but not mast cells derived *in vitro* from *W* mutant mice and that also promoted the development of the erythroid burst-forming unit (BFU-E) in synergy with interleukin-3 (IL-3). Based on these findings, Nocka *et al.* (1990b) proposed that KL was the ligand for the receptor encoded at c-*kit*. Shortly thereafter, Nocka *et al.* and two other groups simultaneously reported the cloning and characterization of a novel growth factor (herein designated SCF) that represented a product of the *Sl* locus and a ligand for the receptor encoded at c-*kit* (Williams *et al.*, 1990; Copeland *et al.*, 1990; Martin *et al.*, 1990; Zsebo *et al.*, 1990a,b; Huang *et al.*, 1990; Anderson *et al.*, 1990). Notably, all three groups used mast cell proliferation *in vitro* as their bioassay for the growth factor or in the characterization of the factor's spectrum of bioactivity.

Physical characterizations of the natural and recombinant SCF products indicate that they are heavily glycosylated, with O-linked and N-linked sugars, and exist as noncovalently associated dimers (Arakawa *et al.*, 1991; Lu *et al.*, 1991; Langley *et al.*, 1992). The soluble

form of the SCF-1 protein, which comprises the first 164 or 165 aa of the extracellular domain, is probably derived from the larger cell-associated precursor by proteolysis in a fashion analogous to the derivation of the secreted forms of CSF-1 or TGF-α from their membrane-associated precursors (Anderson *et al.*, 1990; Huang *et al.*, 1990; Martin *et al.*, 1990; Zsebo *et al.*, 1990a,b; Massagué, 1991; Lu *et al.*, 1992).

Two alternatively spliced forms of human SCF have been reported (Anderson *et al.*, 1991) (Fig. 3). The full-length mRNA encodes a larger protein species (SCF-1) of 248 amino acids, which is a transmembrane protein comprising a 25-aa cleaved signal peptide, a 189-aa extracellular ligand domain that includes a proteolytic cleavage site, a hydrophobic 21- to 23-aa membrane-spanning region and a short (36–37 aa) intracellular domain. The alternative SCF mRNA lacks exon 6, a deletion of 84 base pairs (bp). This shorter mRNA species gives rise to a protein (SCF-2) that is also membrane associated but that lacks 28 amino acids, including one of the four potential N-linked glycosylation sites and the C terminus (Ala-164 and Ala-165) of the soluble SCF, as well as the protease recognition site (Anderson *et al.*, 1990, 1991). Because this form of the protein yields soluble SCF less efficiently than the longer form of the transmembrane protein, regulation of the abundance of the alternatively spliced messages might importantly contribute to the regulation of the production of soluble and/or membrane-associated SCF by the cell. Notably, the biological effects of the membrane-bound (as opposed to soluble) forms of the protein may be significantly different, at least with respect to bone marrow progenitor cells (Toksoz *et al.*, 1992). Three alternatively spliced forms of SCF mRNA have been reported in the mouse: (1) the full-length form; (2) a species lacking exon 6, which, like the corresponding human transcript, produces a 28-amino-acid deletion; and (3) an alternative form with a smaller 16-amino acid deletion of the exon 6 sequence (Anderson *et al.*, 1990; Flanagan *et al.*, 1991), extending C terminally to the C-terminal alanine residue 164 of the soluble kit ligand (Lu *et al.*, 1991).

Most studies of SCF function have focused on the biological activity of either soluble SCF or the extracellular domain of the membrane-associated form of the receptor. However, the Sl^{17H} allele, which produces a splicing defect resulting in a cytoplasmic tail of 28 (not 36) amino acids, with only the first amino acid read in the correct frame, has phenotypic effects that include sterility in male but not female Sl^{17H}/Sl^{17H} mice (Brannan *et al.*, 1992). These findings suggest that the cytoplasmic tail of the transmembrane form of SCF has an important biological function that has yet to be defined.

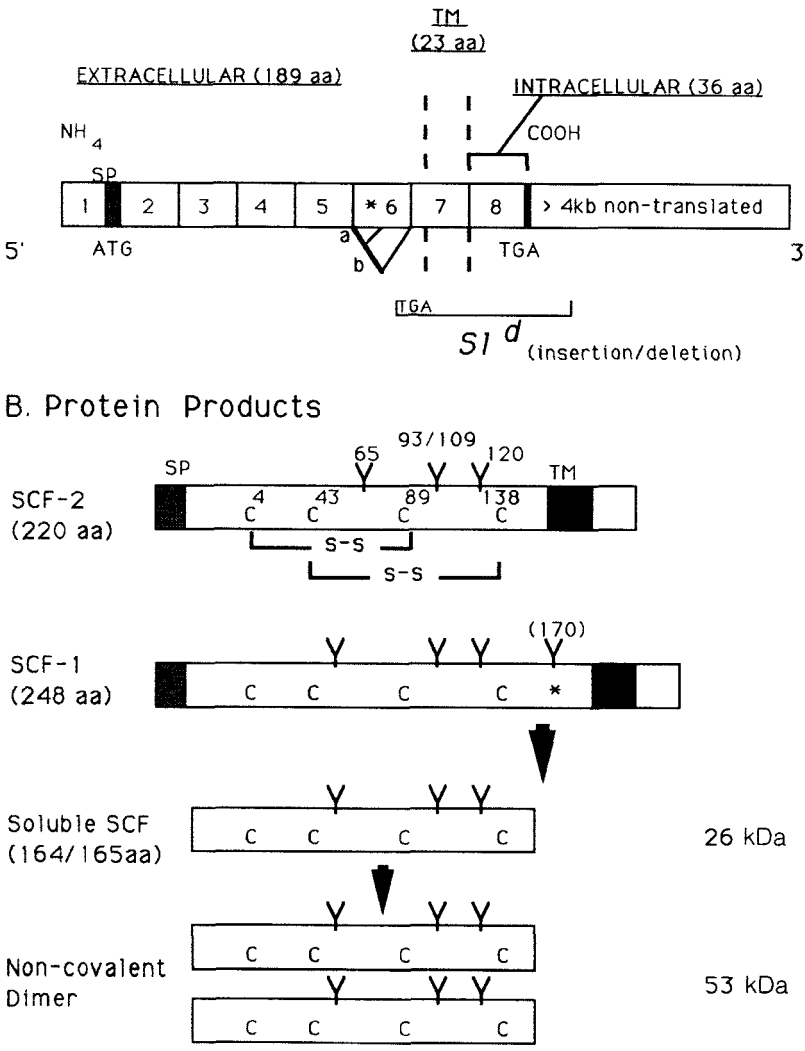

FIG. 3. Structure of the *SCF* gene and SCF. (A) Since the original description by Martin *et al.* (1990), little more has been published concerning the exon/intron structure of the *SCF* gene. The promoter has not yet been characterized. We show a schematic map (not drawn to scale) depicting the locations of the exonic regions in the cDNA that encode the full-length membrane-associated SCF products, SCF-1 or SCF-2. The location of the ATG and signal peptide coding sequences in exon 1 (provisional) are shown; in exon 6 of the cDNA and in the corresponding peritransmembrane region of the SCF translation products, an asterisk indicates the location of a site of protease cleavage that gives rise to the soluble form of the factor; letters a and b indicate the location of two alternative splicing events that give rise to alternative transcripts that lack part (a) or all (b) of exon 6, which encodes the SCF-2 product, which lacks the peptidase cleavage site (Flanagan *et al.*, 1991; Anderson *et al.*, 1991). The position of the insertion/deletion that

B. Genomic Organization and Chromosomal Location of SCF

In the mouse, *Sl* maps on chromosome 10 between the genes *Peptidase-2* (*Pep-2*) and *phenylalanine hydroxylase* (*Pah*), which map on the distal end of the long arm of human chromosome 12 (Ropers and Craig, 1989). Thus, the *Sl/SCF* locus appeared to be part of the conserved linkage group that maps on the long arm of human chromosome 12. However, mouse chromosome 10 also contains genes that map to three other human chromosomes. Therefore, we directly investigated the chromosomal location of *SCF* in man and reported that *SCF* maps on human chromosome 12, between 12q14.3 and 12qter (Geissler *et al.*, 1991). This study and others published subsequently (Anderson *et al.*, 1991; Mathew *et al.*, 1992) localized the human homologue of *Sl* to 12q22–12q24, and provided further evidence for genetic conservation between the distal portion of mouse chromosome 10 and the distal end of the long arm of human chromosome 12 (Fig. 4).

The location of the transcription initiation site, the promoter, and the detailed intron/exon structure of the *SCF* gene have not yet been reported. However, in their original description of the cloning of *SCF*, Martin *et al.* (1990) reported the locations of introns in the coding regions of the human and rat *SCF* genes. In both species, the *SCF* gene is composed of at least 8 exons ranging in size from 51 to more than 183 bp in length (Martin *et al.*, 1990). The locations of introns in the coding region of *SCF* are conserved in rats, mice, and humans (Anderson *et al.*, 1990; Martin *et al.*, 1990). Exon 1 encodes more than 198 bp of 5′ untranslated sequence and the first five amino acids of the putative 25 amino acid signal peptide. Exons 2–7 encode portions of the extracellular domain of the ligand, exon 7 encodes the transmembrane region, and exon 8 encodes 35/36 amino acids in the cytoplasmic tail, the stop codon, and part or all of the very long (~4 kb) 3′ untranslated region of the transcript (Martin *et al.*, 1990). As noted previously, two alternative murine *SCF* transcripts that have been identified lack the sequences encoding the proteolytic cleavage site (Anderson *et al.*,

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introduces a non-sense (TGA) codon in the Sl^d allele is shown (Zsebo *et al.*, 1990b; Copeland *et al.*, 1990; Flanagan *et al.*, 1991; Brannan *et al.*, 1991; Mathew *et al.*, 1992). (B) The structure of the SCF protein products, their sizes, the locations and numbers of cysteine residues that are involved in intramolecular disulfide bonds (indicated), or sites of N-glycosylation (Y) or potential N-glycosylation (170) (Martin *et al.*, 1990; Anderson *et al.*, 1990; Huang *et al.*, 1990; Arakawa *et al.*, 1991; Flanagan *et al.*, 1991; Lu *et al.*, 1991; Langley *et al.*, 1992). All characteristics depicted are conserved between rat and human SCF, except for one site of N-glycosylation, which occurs at amino acid 93 in the human and at amino acid 109 in the rat. Note: all amino acids are numbered based on their position in the mature peptide.

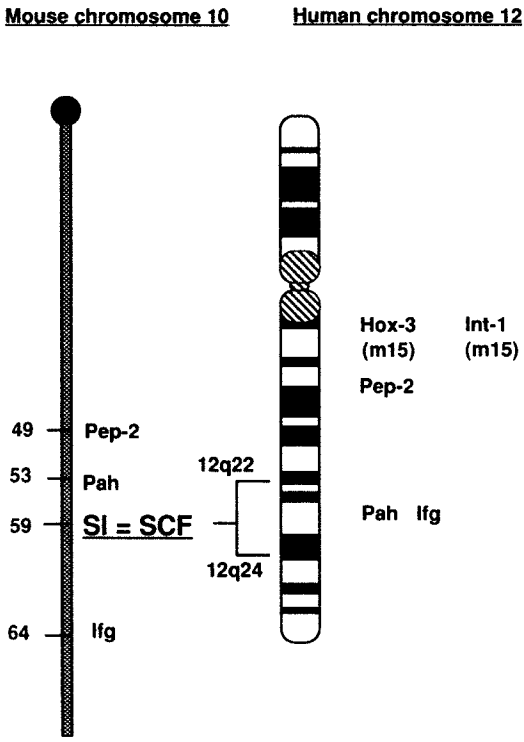

FIG. 4. Location of *SCF* in mouse and human genomes. A partial linkage map of mouse chromosome 10 is shown with the approximate distances of the listed loci from the centromere indicated on the left (Hillyard *et al.*, 1992). A cytogenetic map of human chromosome 12 is shown with the approximate locations of several representative loci (Craig and McBride, 1991). The loci that are included in the region of homology between these chromosomes include those for peptidase (*Pep-2*), phenylalanine hydroxylase (*Pah*), stem cell factor (*SCF*) (Geissler *et al.*, 1991; Anderson *et al.*, 1991), and interferon -γ (*Ifg*). The homeobox gene *Hox-3* and the oncogene *Int-1*, which map on human 12q proximal to *Pep-2*, map on chromosome 15 in the mouse and therefore define the proximal boundary of the region of homology between these two chromosomes.

1990; Flanagan *et al.*, 1991). One of these is missing 84 nucleotides relative to the full-length mouse SCF cDNA (Anderson *et al.*, 1990; Huang *et al.*, 1990; Flanagan *et al.*, 1991). The end points of the missing sequence correspond to the boundaries of exon 6 reported for the rat and human *SCF* genes (Martin *et al.*, 1990). The deletion in the second variant is smaller (48 bp) but shares the same 5′ boundary (Anderson *et al.*, 1990). Therefore, these transcripts are almost certainly derived from the use of alternative 3′ splice acceptor sites in the precursor mRNAs.

Despite the apparent evolutionary relationship between the SCFR and CSF-1R receptors and their genes, c-*kit* and c-*fms*, the homology between their respective ligands has been difficult to detect. However, Bazan (1991) reported that an evolutionary relationship between SCF and CSF-1 could be appreciated by registering equivalent exons and regions that encode similar secondary structures. Based on such findings, Bazan speculated that the interaction of SCF with the SCFR may resemble the interactions of helical cytokines with the hematopoietic receptor superfamily.

IV. Tissue Distribution of SCF and the SCF Receptor

The patterns of expression of c-*kit* and *SCF* during mouse embryogenesis support the hypothesis that these genes are involved in regulating the migration and spatial distribution of cells in the hematopoietic, melanoblast, and germ cell lineages, as well as in the proliferation and differentiation/maturation of these cells at their definitive sites in the developing embryo (Matsui *et al.*, 1990; Orr-Urtregger *et al.*, 1990; Keshet *et al.*, 1991). However, the patterns of *SCF* and c-*kit* expression clearly extend beyond those tissues in which *W* or *Sl* mutations have reported phenotypic effects, and indicate that the SCFR signaling pathway may also be important in the development of cells in the placenta, the nervous system, the septa of the heart, the lung, the facial chondrogenic nuclei, and the midgestational kidney.

Examination of *SCF* and c-*kit* expression in the genital ridges of 10-day embryos at the onset of colonization by migrating primordial germ cells revealed inverse gradients of expression along the route of migration between the dorsal mesentery and the gonad anlage, with *SCF* expression highest in the genital ridge and c-*kit* expression highest in the dorsal mesentery. By gestation day 11.5, after colonization of the gonad is completed, *SCF* expression along the migratory route ceases but remains high in the gonad throughout sexual differentiation.

In the adult ovary expression of c-*kit* is confined to the oocyte (Arcesi *et al.*, 1992; Orr-Urtreger *et al.*, 1990; Keshet *et al.*, 1991) and *SCF* is expressed at highest levels in the surrounding granulosa cells (Keshet *et al.*, 1991). This pattern of expression is consistent with the hypothesis proposed by several groups that cells lining the migratory route may guide the primordial germ cells by expressing the membrane-associated form of SCF, whereas expression of *SCF* in the gonad anlage may indicate a chemotactic role for SCF in directing the homing of germ cell precursors (Flanagan and Leder, 1990; Williams *et al.*, 1990;

Matsui *et al.*, 1990; Flanagan *et al.*, 1991; Keshet *et al.*, 1991). The expression of c-*kit* and *SCF* in the developing ovarian follicle during postnatal life suggests that interactions between the SCFR and its ligand may also be involved in oocyte maturation and/or survival.

Investigations into the expression of c-*kit* and *SCF* in the skin during melanocyte migration are also consistent with a chemotactic/haptotactic role for SCF in the development of pigmentation (Keshet *et al.*, 1991). Prior to melanocyte invasion, *SCF* expression can be detected in the subdermal mesenchymal cells of the limb buds; such *SCF* expression persists during and even after melanocyte colonization. During embryogenesis in the mouse, expression of c-*kit* in the skin appears to be confined to melanocyte precursors and mast cells and persists in melanocytes during their proliferation and differentiation (Keshet *et al.*, 1991). However, studies in humans indicate that, under some circumstances, keratinocytes may also express SCF (Longley *et al.*, 1993).

Both *SCF* and c-*kit* are also expressed during early brain development (Matsui *et al.*, 1990; Orr-Urtreger *et al.*, 1990). Specifically, *SCF* is expressed in the floor plate of the neural tube between days 12 and 15 of gestation and subsequently in the ventrolateral regions associated with motor neuron differentiation. c-*kit* expression has been noted in migrating neural crest cells and in the differentiating neurons in the dorsal neural tube and dorsal ganglia (Keshet *et al.*, 1991). This pattern of expression of *SCF* and c-*kit* suggests that SCF may also function as a chemoattractant for neurons and/or axonic processes. c-*kit* and *SCF* are also expressed at high levels in the central nervous system of the adult mouse (Qiu *et al.*, 1988; Geissler *et al.*, 1988b) and rat (Morii *et al.*, 1992) (see Section XIII).

At mouse gestation day 11.5, c-*kit* is highly expressed in the liver, which is at the peak of its hematopoietic activity (Orr-Urtreger *et al.*, 1990; Keshet *et al.*, 1991). In the adult hematopoietic system, c-*kit* mRNA expression appears to be most prominent in those lineages that are influenced by *W* mutations (André *et al.*, 1989; Nocka *et al.*, 1989). c-*kit* mRNA is expressed in mast cells and in early erythroid and myeloid cell lines (André *et al.*, 1989; Nocka *et al.*, 1989). Autoradiographic studies assessing the binding of ^{125}I-labeled SCF indicate that the SCFR is widely distributed among the bone marrow cells of adult mice, particularly on blasts, promyelocytes/myelocytes, promonocytes, monocytes, eosinophilic myelocytes, eosinophils, and some lymphocytes (Metcalf and Nicola, 1991). However, the number of receptors per cell varies extensively according to cell type, with blasts and some lymphocytes exhibiting the highest levels of labeling (Metcalf and Nicola, 1991). Recently, Ogawa *et al.* (1991) provided

evidence that depletion of adult mouse bone marrow cells that express the SCFR resulted in virtual elimination of hematopoietic progenitor cells responsive to IL-3, GM-CSF, or M-CSF, and also virtually eliminated CFU-S. Early evidence indicated that human basophils lacked detectable expression of the SCFR (Valent and Bettelheim, 1990; Lerner *et al.*, 1991). However, Columbo *et al.* (1992) show that some human blood basophils may express small amounts of the SCFR.

In general, analysis of the distribution of SCFR mRNA and protein on hematopoietic cells indicates that levels of SCFR are highest during early stages of development and progressively diminish in parallel with maturation of the various hematopoietic lineages. The mast cell represents an exception, in that mature mast cells both express the SCFR and exhibit responsiveness to SCF (see Section X).

Analyses of various cell populations *in vitro* indicates that there are multiple potential cellular sources of SCF during lymphohematopoiesis. SCF is expressed by mouse fetal liver-derived stromal cells (Zsebo *et al.*, 1990b), bone marrow stromal cells derived from mice (McNiece *et al.*, 1991a) or humans (Aye *et al.*, 1992), and certain thymic stromal cells (N. Williams *et al.*, 1992), as well as by 3T3 fibroblasts (Anderson *et al.*, 1990; Flanagan and Leder, 1990; Nocka *et al.*, 1990b; Williams *et al.*, 1990; Zsebo *et al.*, 1990b). Vascular endothelial cells have been identified as another potential source of SCF (Aye *et al.*, 1992). The soluble form of the molecule is made abundantly, and is present in the peripheral blood of humans in the nanogram/milliliter range (Langley *et al.*, 1993). On the other hand, very little is known about the tissue levels of membrane-associated or soluble forms of the SCF protein produced *in vivo*, or how such production is regulated.

V. Nature and Biological Consequences of Mutations Affecting SCF or the SCF Receptor

A. Mice

A large number of independent mutations have been described at both *W* and *Sl* loci (for reviews, see Silvers, 1979a; Russell, 1979). In fact, a spontaneous mutation study at the Jackson Laboratory concluded that the mutation rate at *W* was much higher than at other loci that have dominant, grossly observable manifestations (Schlager and Dickie, 1967). Over 30 X-ray-induced *Sl* mutations have been described at Oak Ridge and at Harwell (Silvers, 1979a) and at least five spontaneous *Sl* mutations were reported over a 3-year period at the Jackson Laboratory (Russell and Bernstein, 1966).

In general, a double dose of mutant *W* or *Sl* alleles (*W**/*W** or *Sl**/*Sl**) produces a hypoplastic macrocytic anemia, a lack of skin pig-

mentation, sterility, and a profound mast cell deficiency (Geissler and Russell, 1983a,b; Kitamura *et al.*, 1978; Russell, 1979; Kitamura and Go, 1979; Kitamura, 1989); the *W* or *Sl* alleles with the most severe phenotypic effects, when homozygous, produce death *in utero* or in the immediate perinatal period due to severe anemia (Russell, 1979). *W*/W** or *Sl*/Sl** mice can exhibit reductions in numbers of early hematopoietic cells (such as CFU-S), bone marrow granulocyte precursors and megakaryocytes, and certain early B cell forms, but numbers of circulating granulocytes and platelets, and spleen and bone marrow B cells, are generally normal, probably because of mechanisms that compensate for the deficiencies in early stages of these lineages (Russell, 1979; Geissler and Russell, 1983a) (see Section IX,B). Moreover, B or T cell function in these mice appears to be normal (reviewed in Galli *et al.*, 1992). Animals that are heterozygous for mutant *W* or *Sl* alleles (*W*/+* or *Sl*/+*) exhibit a pattern of coat depigmentation (spotting) that can be characteristic for each allele but that generally comprises an abdominal spot, a spot on the forehead, and variable depigmentation of the tips of the extremities and the tail. However, *W*/+* or *Sl*/+* mice are fertile and exhibit no or only modest reductions in red cell or mast cell numbers (Russell, 1979; Silvers, 1979a; Kitamura, 1989).

The mutant *Sl* alleles that have the most dramatic effects, generally resulting in death *in utero* or shortly after birth, are due to deletions that produce complete loss of SCF function. Such alleles include *Sl* (Sarvella and Russell, 1956), as well as Sl^{J}, Sl^{gb}, Sl^{8H}, Sl^{10H}, Sl^{12H}, and Sl^{18H} (Russell, 1979; Silvers, 1979a; Copeland *et al.*, 1990; Huang *et al.*, 1990; Zsebo *et al.*, 1990b). In contrast, mice homozygous for the *Steel-Dickie* (Sl^{d}) allele are viable, despite exhibiting a moderately severe macrocytic anemia, a complete lack of skin pigmentation, a profound mast cell deficiency, and sterility (Bernstein, 1960; Kitamura and Go, 1979; Russell, 1979; Silvers, 1979a). The less severe effects of the Sl^{d} allele on viability suggested that this mutation might preserve some SCF function. We showed that the Sl^{d} allele encodes an abundant but smaller than normal SCF transcript and is associated with a novel *SCF* DNA restriction fragment length polymorphism (RFLP) (Zsebo *et al.*, 1990b). Subsequently, Flanagan *et al.* (1991), Brannan *et al.* (1991), and Huang *et al.* (1992) showed that the Sl^{d} allele represents an intragenic insertion/deletion that removes 242 bp at the 3′ end of the coding sequence. The truncated Sl^{d} transcript encodes almost all of the extracellular domain of SCF but lacks coding sequences for both the transmembrane and the intracellular domains of the wild-type protein. Notably, expression of the Sl^{d} cDNA in transfected COS cells

results in secretion of soluble biologically active product that is capable of inducing mast cell proliferation, but that fails to result in the appearance of a membrane-associated form of SCF (Flanagan *et al.*, 1991).

Although it is not yet clear to what extent soluble SCF is produced by Sl/Sl^d or Sl^d/Sl^d mice *in vivo*, the phenotypic abnormalities of these mice, together with analyses of the biological activity of recombinant forms of the Sl^d product, suggest that a membrane-associated form of SCF is necessary for a normal phenotype *in vivo*. Toksoz *et al.* (1992) generated cell lines that stably expressed soluble or membrane-associated forms of SCF. They found that cell lines expressing membrane-associated SCF supported the long-term production of primitive hematopoietic progenitors *in vitro*, whereas cells that exclusively produced soluble SCF only transiently maintained hematopoiesis. In demonstrating a critical role in hematopoiesis for the membrane-associated form of SCF, Toksoz *et al.* (1992) provided a molecular explanation for previous work showing that when normal (+/+) splenic tissue was transplanted to *Steel* mice, enhanced hematopoiesis occurred only in the donor spleen (Bernstein, 1970).

On the other hand, analysis of the effects of recombinant soluble SCF in Sl/Sl^d mice indicates that the provision of high levels of soluble SCF can partially reverse some of the hematological abnormalities expressed by these animals (Zsebo *et al.*, 1990b). Thus, treatment of Sl/Sl^d mice with polyethylene glycol-modified rrSCF^{164} for 21 days at 30 μg/kg/day, subcutaneously (s.c.), not only markedly improved the animals' hematocrits but also repaired the dermal mast cell deficiency at the s.c. injection site (Table I). Indeed, the density of dermal mast cells at rrSCF^{164} injection sites in Sl/Sl^d mice exceeded by approxi-

TABLE I
EFFECT OF TREATMENT WITH RECOMBINANT RAT SCF^{164} ON HEMATOCRIT AND DERMAL MAST CELL NUMBERS IN WCB6F$_1$-Sl/Sl^d MICE[a]

Treatment	Hematocrit (%)		No. mast cells/mm^2 of dermis
	Before treatment	After treatment	
SCF (30 μg/kg/day)	27.3 ± 2.6	40.9 ± 4.3[b]	77.9 ± 16.9[b]
Vehicle	28.6 ± 1.4	25.9 ± 5.4	0 ± 0

[a] Data are for a 3-week treatment period. Reproduced from Zsebo *et al.* (1990b) with permission.
[b] $p < 0.001$ versus value for vehicle-injected group.

mately twofold the density of mast cells in the dermis of the congenic normal (+/+) mice.

Notably, some *Sl* mutations produce sterility in only one sex. Sl^{pan}, Sl^{t}, and Sl^{con} produce female sterility in the homozygous state (Beechey and Searle, 1983, 1985; Kuroda *et al.*, 1988) whereas Sl^{17H} produces male sterility in the homozygous state (Peters *et al.*, 1987). These mutations have relatively mild phenotypic effects (e.g., Sl^{17H}, Sl^{pan}, Sl^{t}, and Sl^{con} homozygotes have mild anemia and exhibit some wild-type coat pigmentation, suggesting that the mutations may produce only limited impairment of SCF function. Indeed, Brannan *et al.* (1992) showed that Sl^{17H} has a T → A transversion within the polypyrimidine tract immediately upstream of the AG dinucleotide defining the 5′ boundary of exon 8 of *SCF;* this produces a splicing defect that results in the skipping of exon 8 and fusion of exon 7 to exon 9. This, in turn, results in an SCF cytoplasmic tail that is shortened by 8 amino acids and that has only the first amino acid read in the correct frame. The SCF encoded by the Sl^{17H} allele exhibits an activity in a mast cell proliferation assay that is very similar to that of wild-type SCF. Taken together, these findings indicate that the cytoplasmic tail of the membrane-associated SCF may have important biological function(s).

To date a total of 14 *W* mutations have been shown to be associated with alterations in the c-*kit* structural gene, comprising both regulatory and structural mutations; several are single base pair substitutions in the region encoding the kinase domain, and these confer a partial or complete loss of SCFR kinase activity as assessed by *in vitro* kinase assays (Geissler *et al.*, 1988b; Bernstein *et al.*, 1990; Nocka *et al.*, 1990a; Reith *et al.*, 1990, 1991; Tan *et al.*, 1990).

All *W* mutations behave in a codominant fashion with other *W* alleles. However, even though most *W* mutations influence melanogenesis, gametogenesis, hematopoiesis, and mast cells to similar degrees, some mutations influence only one or two of the usually affected tissues. For example, when W^{44} is homozygous, pigment and germ cell development are affected but not erythropoiesis, and W^{41}/W^{41} mice are anemic, predominantly white, but fertile. These exceptions provided strong evidence that the *W* locus tissue defects were genetically separable and developmentally independent (Geissler *et al.*, 1981). However, the mechanisms by which certain *W* mutations have severe effects on only one or two of the typically affected tissues are not understood. The phenomenon may reflect either differences in the effects of *W* mutations on the quantity or quality of c-*kit* expression in different lineages, for example, by differentially influencing the rate of c-*kit* transcription in different tissues, or tissue-specific differences

in the threshold of SCFR function required for the normal development of the different cell lineages. Tissue-specific differences in the threshold of wild-type SCFR function which is required for normal development could be determined by the level of expression of the SCF ligand in the microenvironment, by the relative concentrations of substrates involved in the c-*kit* signal transduction pathway in each lineage, the specific effects of each c-*kit* mutation on binding affinities for these substrates, as well as by differences in the expression of redundant RTK signaling pathways in the tissues.

Some *W* mutations exhibit anomalies with respect to the relative severity of their phenotypic effects in the heterozygous (*W**/+) or homozygous (*W**/*W**) mutant mice. Specifically, the heterozygous phenotype of alleles that are homozygous viable are often more severe than those involving *W* alleles that are homozygous lethal. This phenomenon was originally noticed in 1949 by Elizabeth Russell in her detailed comparison of the phenotypic effects of the first two *W* mutations to be described, *W* and W^v (Russell, 1949), and was subsequently shown to be characteristic of a large number of independent mutations at *W* (Geissler *et al.*, 1981). The discovery that *W* encodes the SCFR has shed considerable light on the nature of some of these "dominant negative" effects.

Alleles that exhibit strong, dominant negative effects comprise mutations at distinct sites in the kinase domain of the c-*kit* gene; these mutations result in the production of SCFRs with reduced stability and/or reduced intrinsic kinase activity but that are nevertheless expressed on the cell surface. For example, the original *W* mutation, which has relatively mild heterozygous effects but which is homozygous lethal, represents a point mutation at an exon/intron junction that causes improper mRNA processing and exon skipping that results in the production of a truncated SCFR lacking the transmembrane domain (Hayashi *et al.*, 1991). Consequently, the product of the *W* allele is not expressed on the cell membrane and does not undergo autophosphorylation when stimulated with SCF (Reith *et al.*, 1990). In contrast, the recessive lethal W^{37} (Nocka *et al.*, 1990a; Reith *et al.*, 1990) and W^{42} (Tan *et al.*, 1990) mutations, which have relatively severe heterozygous effects on pigmentation (W^{37} or W^{42}) and/or hematopoiesis (W^{42}), comprise point mutations in the tyrosine kinase domain of the SCFR that result in amino acid substitutions (W^{37}:$Glu^{582} \rightarrow$ Lys; W^{42}:$Asp^{790} \rightarrow$ Asn) that destroy its intrinsic kinase activity.

W^{42} is the most strongly dominant of all mutant *W* alleles described to date (Geissler *et al.*, 1981). Moreover, the exogenous expression of

the W^{42} c-*kit* cDNA during the development of transgenic mice successfully recapitulates certain aspects of the *W* mutant phenotype (Ray *et al.*, 1991). W^{42} comprises a missense mutation in an essential amino acid of the SCFR kinase domain, which abolishes the kinase activity of the protein but does not affect its normal expression on the cell surface (Tan *et al.*, 1990). Together these findings suggest that the W^{42} product exhibits trans-dominant negative interference on the function of the wild-type receptor, probably by interfering with the normal function of the dimerized receptor. Signal transduction through the SCFR, as with the CSF-1R and PDGFR (Ullrich and Schlessinger, 1990), involves receptor dimerization and internalization following ligand binding (see Section VII). It therefore appears likely that "dominant negative" effects involve interference by the mutant form of the receptor on the function of the wild-type counterpart. At least part of this interference appears to be mediated by interactions at the cell surface. This could involve competition for limiting amounts of ligand and perhaps by the inactivation of the wild-type receptor molecules in heterodimers involving the mutant protein. However, unlike the product of the W^{42} allele, which is expressed normally on the cell surface (Tan *et al.*, 1990), the product of the W^{37} allele is intrinsically less stable and is only weakly expressed at the cell surface (Nocka *et al.*, 1990a). Accordingly, the basis for the relatively severe dominant negative effect of this allele on pigmentation is not yet understood.

The homozygous viable mutations W^{41} ($Val^{831} \rightarrow$ Met), W^{v} ($Thr^{660} \rightarrow$ Met), and W^{55}, a remutation to W^{v}, also map in the tyrosine kinase domain, but, in comparison to W^{42}, produce less severe reductions in the levels of intrinsic kinase activity expressed by the corresponding SCFR proteins, which are expressed normally at the cell surface (Nocka *et al.*, 1990a; Reith *et al.*, 1990). The relative severities of the kinase deficiencies exhibited by these mutant forms of the SCFR correlate well with the severities of the abnormal phenotypes induced by these mutations *in vivo*. The same correlation has been noted for the capacity of alternative forms of the mouse and human SCFR to associate with cytoplasmic protein substrates, such as phospholipase C-γ1 and phosphatidylinositol 3′-kinase (Nocka *et al.*, 1990a; Reith *et al.*, 1990; Herbst *et al.*, 1992). Thus, the W^{37} product does not associate with P13′K or PLC-γ1 following exposure to SCF, whereas the product of the W^{41} allele, which has relatively mild phenotypic effects, retains a significant capacity to associate with these cytoplasmic substrates.

Recently, three additional *W* mutations, W^{Jic}, W^{n}, and W^{f}, were found to comprise point mutations in the tyrosine kinase domain of c-*kit* (Larue *et al.*, 1992; Tsujimura *et al.*, 1993). The W^{Jic} mutation

results in the substitution of Gly-595 in the ATP-binding site, which inactivates the kinase activity of the protein. The W^n allele is transcribed normally but a substitution of Ala-835 impairs post-translational processing of the protein and prevents the expression of detectable levels of receptor at the cell surface. W^f, which produces relatively mild phenotypic effects closely resembling those of W^{41}, results in the substitution of Arg-816 in the kinase domain, which only mildly impairs the intrinsic kinase activity of the receptor (Larue *et al.*, 1992; Tsujimura *et al.*, 1993).

B. Rats

Germ-line c-*kit* mutations have been discovered in other species. Tsujimura *et al.* (1991) reported a spontaneous $W^{c\text{-}kit}$ mutation in the rat (*Ws*) which, in the homologous state, produces a phenotype similar, but not identical, to that of the W/W^v mouse (Niwa *et al.*, 1991). *Ws/Ws* rats are black-eyed, white, anemic, and profoundly mast cell deficient (Niwa *et al.*, 1991; Onoue *et al.*, 1993). However, the anemia improves with age and the rats are fertile. Molecular characterization of the c-*kit* transcript expressed in the tissues of the *Ws/Ws* rat identified a deletion of 12 nucleotides encoding four amino acids near the tyrosine autophosphorylation site in the kinase domain of the receptor (Tsujimura *et al.*, 1991).

C. Humans

The piebald trait in man is an autosomal dominant congenital syndrome associated with patches of apigmented skin and hair that are distributed in a pattern reminiscent of the *W*/+ mouse, i.e., white spots on the abdomen, extremities, and forehead (Hulten *et al.*, 1987). Based on the presence of congenital chromosome 4 deletions in some piebald individuals, the locus controlling this trait was provisionally mapped between 4q11–4q12 (Lacassie *et al.*, 1977), colocalizing this trait with the c-*kit* structural gene. The hypothesis that piebaldism may arise from aberrant c-*kit* function was recently confirmed by two groups. Fleischman *et al.* (1991) identified a cytogenetically normal piebald individual that carried a hemizygous deletion that included c-*kit* and the neighboring *PDGFR-α* gene. More definitive proof was provided by reports that other piebald individuals carry point mutations causing missense amino acid substitutions in the SCFR kinase domain, specifically, codon 664 glycine (Giebel and Spritz, 1991), codon 584 phenylalanine (Spritz *et al.*, 1992a,b), and codon 583 glutamic acid (Fleischman, 1992a,b), or exhibit one of two frameshift mutations that map in codons 642 and 561 (Spritz *et al.*, 1992a).

No hematological abnormalities were detected in piebald individuals with c-*kit* mutations, nor in the previously reported cases of piebaldism, which include one presumed homozygote (Hulten *et al.*, 1987; Spritz, 1992). Perhaps this should not be surprising, because the majority of c-*kit* mutations in the mouse have few if any effects on the peripheral blood parameters of the $W^*/+$ heterozygous mice, and rare alleles, such as W^{44} and W^{sh}, which appear to be regulatory mutations (Geissler *et al.*, 1988b; Tono *et al.*, 1992), have no effect on the peripheral blood even when in the homozygous state.

Because the hematologic abnormalities in patients with Diamond–Blackfan anemia (DBA) resemble those of *W* or *Sl* mutant mice, we and others considered the possibility that germ-line or somatic mutations at c-*kit* or *SCF* may be involved in the pathogenesis of DBA. To evaluate this possibility, we (Drachtman *et al.*, 1992; Abkowitz *et al.*, 1992) and others (Olivieri *et al.*, 1991) searched for abnormalities in the structure or expression of either c-*kit* or *SCF* in patients with DBA. The cumulative data from analyses of almost two dozen patients fail to support the hypothesis that DBA arises from alterations in the genes encoding the SCRF or SCF. Of course, these data do not rule out the possibility that subtle defects in one of these genes may yet be identified in some DBA patients.

VI. Regulation of Expression of SCF and the SCF Receptor

A. The SCF Receptor

At the cellular level, c-*kit* expression can be influenced by a variety of cytokines and stimuli. We and others have reported that the level of c-*kit* mRNA expressed by primary mast cell cultures or mast cell lines is significantly reduced after treatment of the cells with either immunologic or nonimmunologic agents or with hematopoietic growth factors. We reported that treatment of long-term mouse mast cell lines, or primary cultures of bone marrow-derived cultured mast cells (BMCMCs) from normal or W/W^v mice, by phorbol myristate acetate (PMA) or IgE and specific antigen reduced levels of c-*kit* mRNA by more than 50% within 2 hours (Geissler *et al.*, 1990). The down-regulation of c-*kit* mRNA in W/W^v mast cells demonstrates that the *W* and W^v mutations do not interfere with cis-regulatory elements involved in down-regulation of c-*kit* mRNA and also indicates that down-regulation of c-*kit* mRNA does not require a SCFR with normal tyrosine kinase activity.

Sillaber *et al.* (1991) reported that IL-4 can down-regulate c-*kit* expression in normal human $CD34^+$ bone marrow cells, human leuke-

mic myeloid cells, and in the HMC-1 human mast cell leukemia line. IL-3 reportedly down-regulates c-*kit* mRNA expression in murine mast cell and myeloid cell lines (Welham and Schrader, 1992) but has no influence on (Sillaber *et al.*, 1991) or up-regulates (Papayannopoulou *et al.*, 1991) the expression of SCFR by human hematopoietic cells. It is not clear whether the discrepancies in the reported effects of cytokines on c-*kit* expression reflect species differences, cell lineage-specific differences, or differences in the metabolic state or cell cycle activity of the cells at the time of the assay. In fact, there is some evidence that c-*kit* expression may be regulated by the c-*myb* transcription factor, which plays an important role in regulating the cell cycle (Ratajczak *et al.*, 1992a,b) (see Section VI,A).

Analyses of mutant mice have also provided some insights into the regulation of c-*kit* expression. Mutant mice of the *mi/mi* genotype are osteopetrotic due to a monocyte/macrophage defect and are also mast cell deficient (Stevens and Loutit, 1982; Stechschulte *et al.*, 1987). Ebi *et al.* (1990, 1992) reported that bone marrow-derived cultured mast cells derived from *mi/mi* mice, unlike their normal counterparts, failed to proliferate or to exhibit phenotypic changes associated with maturation when cultured on monolayers of normal fibroblasts that express SCF on the cell surface (Ebi *et al.*, 1990) or in medium supplemented with high levels of rSCF (Ebi *et al.*, 1992). Ebi *et al.* (1992) also reported that the expression of *SCFR* mRNA and protein is significantly reduced in *mi/mi* BMCMCs and that treatment of these cells with rSCF induced little if any autophosphorylation of the SCFR. Taken together, these results suggest that the low responsivity of *mi/mi* mast cells to SCF reflects their low level of SCFR expression. The *mi/mi* mast cells also exhibited an altered pattern of expression of mRNA encoding the mast cell proteases MMCP-2 and MMCP-6 (Ebi *et al.*, 1992). Although there is more than one possible explanation for this finding, the data are consistent with the hypothesis that the *mi* locus may play a role in regulating the expression of both c-*kit* and the protease genes, for example, by influencing the production of transcription factors that regulate the transcription of several genes in mast cells. The availability of cloned c-*kit* promoter elements involved in the regulation of c-*kit* expression in mast cells and other c-*kit*-positive cell lineages (Yasuda *et al.*, 1993; described later) should allow this hypothesis to be tested experimentally.

Although the mutation has not yet been characterized at the molecular level, several lines of evidence indicate that W^{sh} represents a regulatory rather than a structural c-*kit* mutation. The phenotypic effects of the W^{sh} mutation are less severe than any other known W mutation.

Complementation studies and genetic linkage analyses indicate that W^{sh} is in fact a *W* allele, and adult W^{sh}/W^{sh} mice are black-eyed and white, yet they are fertile and are not anemic (Russell, 1979; Silvers, 1979a; Lyon and Glenister, 1978). In contrast to the majority of *W* mutant genotypes that elicit effects of corresponding severity on red cell numbers and numbers of cutaneous mast cells (Go *et al.*, 1980; Geissler and Russell, 1983b), W^{sh}/W^{sh} adults exhibit a severe mast cell deficiency despite their normal red cell number (Tono *et al.*, 1992). Mast cells derived *in vitro* from the spleen cells of W^{sh}/W^{sh} mice do not adhere to 3T3 cell monolayers, presumably because of their failure to express the SCFR on their cell surface (Stevens and Loutit, 1982; Tono *et al.*, 1992), and immunoprecipitable SCFR protein was not detected in the cytoplasm of W^{sh}/W^{sh} mast cells and was present at greatly reduced levels in the testes of these mice. Amounts of c-*kit* transcript were also drastically reduced in the testes, spleen, and mast cells of W^{sh}/W^{sh} mice, although relatively normal levels were present in the cerebellum. Therefore, the levels of SCFR protein in the tissues of W^{sh}/W^{sh} mice appear to be primarily regulated at the level of transcription. Notably, the nucleotide sequence of the c-*kit* coding region of cloned cDNAs was found to be completely normal, indicating that W^{sh} probably represents a regulatory mutation (Tono *et al.*, 1992).

To elucidate further the regulation of c-*kit* expression, we cloned and characterized the mouse c-*kit* promoter (Yasuda *et al.*, 1993). Primer extension analysis and RNase mapping experiments were conducted to identify the major c-*kit* transcription initiation site (TIS) employed in several mouse cell types, including BALB/c BMCMCs, factor-dependent and factor-independent mouse mast cell lines PT-18 (Pluznik *et al.*, 1982) and MC/Cl.C57.1 (C57.1) (Young *et al.*, 1987), respectively, and cerebellar tissue of adult C57BL/6J mice. Using each of several c-*kit*-specific primers, we detected products that terminated 58 bp upstream from the A in the translation start codon. In addition, several minor primer extension products, between 1 and 4 bp shorter than the major product, were detected in some experiments. The position of the TIS was confirmed by sequence analysis of 5′ c-*kit* cDNAs cloned by rapid amplication of 5′ terminal cDNA. Together, these data indicated that the major c-*kit* TIS, used by mouse mast cells and c-*kit*-positive cells in the mouse cerebellum, is located 58 bp upstream from the translation start codon, and identified several minor TISs, which map less than 12 bp from this site. This comprised the first precise localization of the c-*kit* TIS in any species.

Genomic DNA clones comprising the 5′ end of the mouse c-*kit* transcript were cloned from a cosmid library. Genomic DNA se-

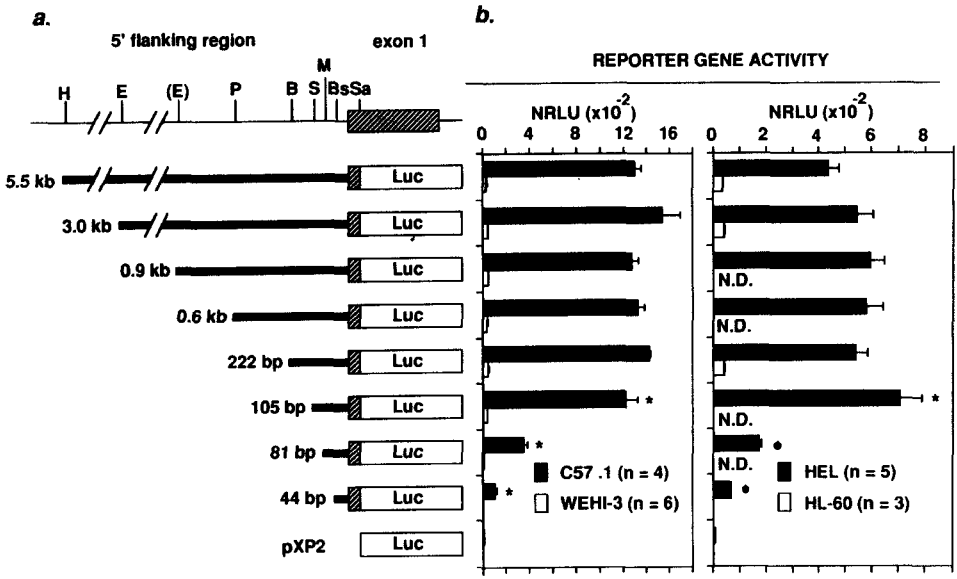

FIG. 5. 5′ Deletion analysis of the mouse c-*kit* promoter. (a) Partial restriction maps of the 5′ end of the mouse c-*kit* gene and reporter gene constructs. Restriction endonuclease sites in the 5′ flanking genomic DNA used to generate the reporter gene constructs are shown. The 5′ flanking genomic DNA region cloned into each construct is represented by a solid bar, extending to the left of the *Sac*I site in the untranslated portion of c-*kit* exon 1, shown by a hatched box. The length of the 5′ flanking region is given on the left in base pairs (bp) or kilobase pairs (kb). H, *Hind*III; E, *Eco*RI; P, *Pvu*III; B, *Bam*HI; S, *Sma*I; M, *Msc*I; Bs, *Bss*HII; Sa, *Sac*I. (b) Luciferase reporter gene promoter assay. Equimolar amounts of luciferase constructs were transiently cotransfected with pCMVhGh into either mouse C57.1 or WEHI-3 cells or human HEL or HL-60 cells. NRLU, the number of sample relative light units (minus background) divided by the total amount of hGH (nanograms) detected in the culture medium. ND, not determined; *, significantly greater than constructs containing shorter, or no, promoter regions ($p < 0.005$). All c-*kit* promoter constructs, which included from 44 bp to 5.5 kb upstream from the major TIS, produced significantly more luciferase activity ($p < 0.005$) in c-*kit*-positive cell lines (C57.1 or HEL) versus the corresponding c-*kit*-negative cell lines (WEHI-3 or HL-60). In a given c-*kit* positive cell line, either mouse C57.1 mast cells or human HEL cells, constructs containing 105 or more base pairs upstream from the major c-*kit* TIS exhibited strong promoter activities that were statistically indistinguishable. Reproduced from Yasuda *et al.* (1993) with permission.

quences upstream from the TIS were subsequently characterized for c-*kit* promoter activity by transiently transfecting c-*kit*-positive or c-*kit*-negative mouse or human cell lines with luciferase reporter gene constructs. As shown in Fig. 5b, all c-*kit* promoter constructs, including from 44 to 5.5 kb upstream from the major TIS, produced signifi-

cantly more luciferase activity ($p < 0.005$) in c-*kit*-positive cell lines (C57.1 or HEL) versus the corresponding c-*kit*-negative cell lines (WEHI-3 or HL-60). Our results also indicated that under our experimental conditions, important cis elements required for cell type-specific expression are contained within 105 bp of the major TIS, in three functionally important subregions: region A between the major TIS and −44 bp, region B between −44 and −81 bp, and region C between −81 and −105 bp. These regions contributed to more than 10-, 3-, and 3-fold increases in luciferase activities, respectively, in c-*kit*-positive cell lines (C57.1 or HEL). In over 5 kb of upstream genomic DNA, we found no evidence for additional elements that significantly influenced luciferase activities.

These data showed that the mouse c-*kit* promoter also functions in human c-*kit*-positive HEL cells, suggesting that important cis elements and trans-acting factors involved in the regulation of c-*kit* expression are likely to be conserved. Figure 6 shows the sequence of the mouse c-*kit* promoter region, and a provisional alignment with the corresponding portion of the human c-*kit* gene (Giebel *et al.*, 1992).

The mouse c-*kit* promoter lacks well-defined promoter elements such as TATA or CCAAT boxes. Nor does it contain recognizable consensus binding-site sequences for the erythroid/mast cell-associated transcription factors GATA-1, GATA-2, or GATA-3 (Zon *et al.*, 1991), even though c-*kit* is expressed in the erythroid and mast cell lineages. A high degree of conservation exists in the region corresponding to the mouse c-*kit* TIS and the contiguous upstream genomic DNA (+1 to −44 bp) that exhibits c-*kit* promoter function in both mouse C57.1 mast cells and human HEL erythroid cells. A conserved potential binding site for the transcription factor AP-2 (Jones *et al.*, 1988) in this region could play a role in the regulation of c-*kit* expression. AP-2 has been shown to be involved in the tissue-specific regula-

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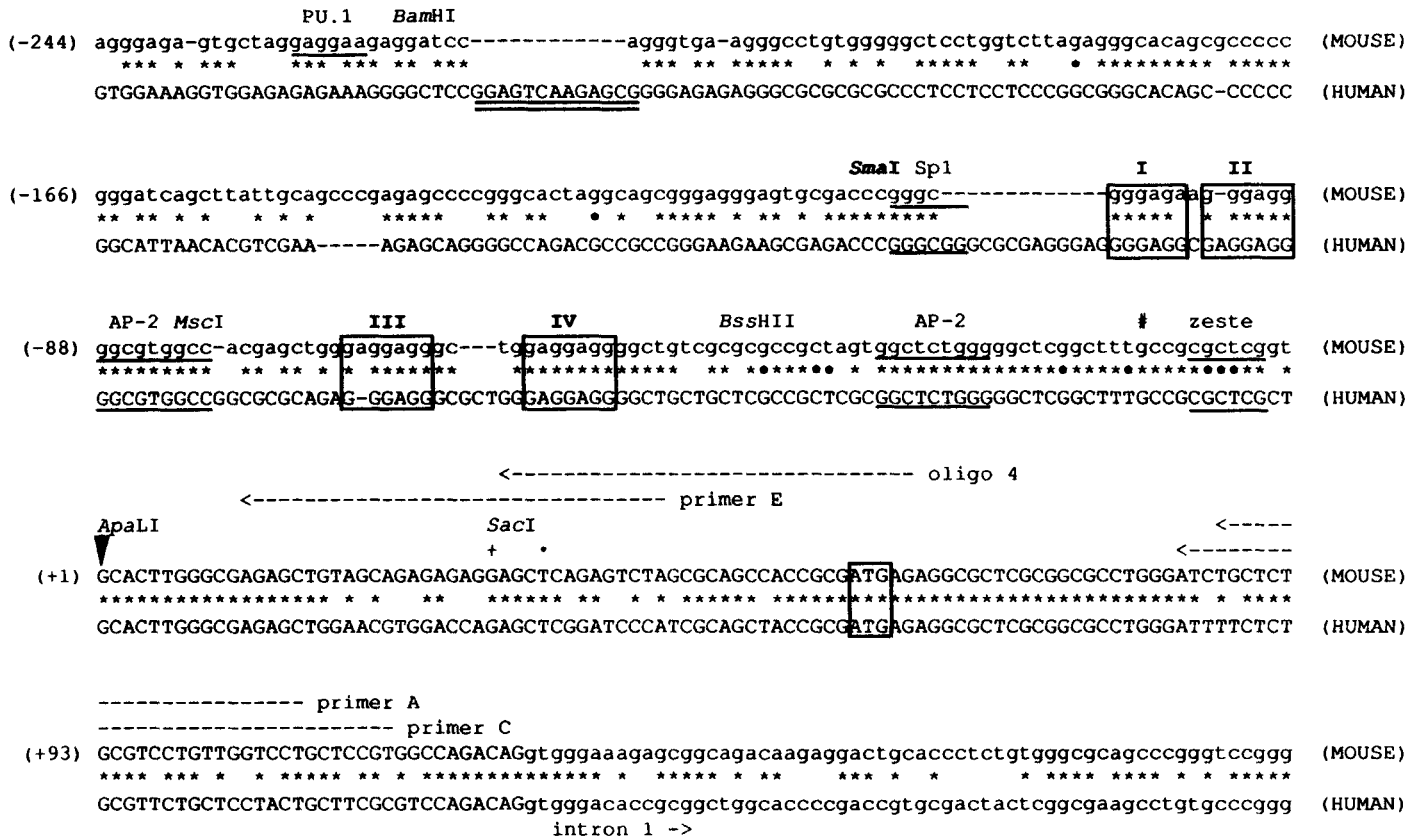
FIG. 6. Nucleotide sequence of the 5′ end of c-*kit* (the transcription initiation sequence = bp + 1). The translation start codon is boxed; exon 1 sequences are shown in capital letters. The exon 1/intron 1 boundary agrees with that reported by Gokkel *et al.* (1992). The complementary sequences of primers A, E, C, and oligo 4 (used in primer extension reactions) are underlined with dashed arrows, indicating their 5′ to 3′ orientation. The end of a previously reported c-*kit* cDNA (+) (Qiu *et al.*, 1988) and the major TIS (▼) and a minor upstream TIS (#) are indicated above the sequence with the corresponding symbols. *Sac*I and other restriction sites used in vector construction are shown. The *Sma*I site at −105 bp (indicated in bold letters) delineates the 5′ end of the region that exhibits maximal promoter activity (see Fig. 5). Potential transcription factor binding sites (Jones *et al.*, 1988) are indicated, and GA-rich regions are double underlined. Reproduced from Yasuda *et al.* (1993) with permission.


PU.1
BamHI
(-244) agggaga-gtgctaggaggaagaggatcc----------agggtga-agggcctgtgggggctcctggtcttagagggcacagcgccccc (MOUSE)
GTGGAAAGGTGGAGAGAGAAAGGGGCTCCGGAGTCAAGAGCGGGGAGAGAGGGCGCGCGCGCCCTCCTCCTCCCGGCGGGCACAGC-CCCCC (HUMAN)
SmaI Sp1
I
II
(-166) gggatcagcttattgcagcccgagagccccgggcactaggcagcgggagggagtgcgacccgggc----------gggagaag-ggagg (MOUSE)
GGCATTAACACGTCGAA-----AGAGCAGGGGCCAGACGCCGCCGGGAAGAAGCGAGACCCGGGCGGGCGCGAGGGAGGGGAGGGCGAGGAGG (HUMAN)
AP-2 MscI
III
IV
BssHII
AP-2
#
zeste
(-88) ggcgtggcc-acgagctgggaggagggc---tggaggagggctgtcgcgcgccgctagtggctctgggggctcggctttgccgcgctcggt (MOUSE)
GGCGTGGCCGGCGCGCAGAG-GGAGGGCGCTGGGAGGAGGGGCTGCTGCTCGCCGCTCGCGGCTCTGGGGGCTCGGCTTTGCCGCGCTCGCT (HUMAN)
oligo 4
primer E
ApaLI
SacI
(+1) GCACTTGGGCGAGAGCTGTAGCAGAGAGAGGAGCTCAGAGTCTAGCGCAGCCACCGCGATGAGAGGCGCTCGCGGCGCCTGGGATCTGCTCT (MOUSE)
GCACTTGGGCGAGAGCTGGAACGTGGACCAGAGCTCGGATCCCATCGCAGCTACCGCGATGAGAGGCGCTCGCGGCGCCTGGGATTTTCTCT (HUMAN)
primer A
primer C
(+93) GCGTCCTGTTGGTCCTGCTCCGTGGCCAGACAGgtgggaaagagcggcagacaagaggactgcaccctctgtgggcgcagcccgggtccggg (MOUSE)
GCGTTCTGCTCCTACTGCTTCGCGTCCAGACAGgtgggacaccgcggctggcaccccgaccgtgcgactactcggcgaagcctgtgcccggg (HUMAN)
intron 1 ->
29

tion of some genes (Leask *et al.*, 1991). Furthermore, during mouse development, AP-2 and c-*kit* are coexpressed in some tissues, including hindbrain, kidney, heart, yolk sac, and some neural crest-derived lineages (Orr-Urtreger *et al.*, 1990; Mitchell *et al.*, 1991).

The interval between −44 and −81 bp, which significantly augments the activity of the mouse c-*kit* promoter in both mouse and human cells, contains a direct repeat of the nonanucleotide GGAGGAGGG (Fig. 6, boxes III and IV). This sequence is similar both to a putative myeloid promoter-specific consensus element (Shapiro *et al.*, 1991) and to a DNase-resistant element (GAGGAGGGG) that is located at a similar position in the promoter of the chicken *β-globin* gene (Emerson *et al.*, 1985). The third functionally important interval, between −81 and −105 bp, contains conserved potential binding sites for AP-2 and Sp1 and two contiguous GA-rich elements (Fig. 6, boxes I and II). The four conserved GA-rich elements in the c-*kit* promoter (Fig. 6, boxes I–IV) share a core consensus sequence (G/A-G-G-A-G-G/A) also found in a noncanonical, functional PU.1-binding site (AAAAGGAGAAG) identified in the human CD11b promoter (Pahl *et al.*, 1993). Therefore, it is possible that one or more of the c-*kit* elements comprise binding sites for PU.1 or other ETS-domain proteins (Karim *et al.*, 1990). A canonical consensus PU.1-binding site is found further upstream in the c-*kit* promoter (Fig. 6), but this site is not well conserved and maps outside the region that is necessary for maximal promoter activity in transfected mouse mast cells or HEL cells. Nevertheless, PU.1 expression does not appear to be sufficient for c-*kit* transcription because both WEHI-3 cells and HL-60 cells express PU.1 (Shapiro *et al.*, 1991; Karim *et al.*, 1990), but neither cell line expresses endogenous c-*kit* mRNA (André *et al.*, 1989; Nocka *et al.*, 1989) nor did either cell line promote the expression of the c-*kit* promoter reporter constructs. It is possible, however, that PU.1 expression is necessary for c-*kit* transcription and that interactions between Sp1 and PU.1 or other ETS-domain proteins are involved in the tissue-specific expression of c-*kit*.

Although the human c-*kit* promoter and TIS have not yet been characterized, the functional promoter analyses described above demonstrate that the mouse c-*kit* promoter comprises three regions that are functionally important in both mouse and human c-*kit*-positive cells. Furthermore, comparison of the nucleotide sequence of the corresponding regions of mouse and human c-*kit* genes shows that the functionally important regions are highly conserved. Together these findings strongly suggest that the location of the c-*kit* TIS and the structure of the promoter of the human c-*kit* gene are likely to be similar or identical to those described here for the mouse gene.

We have shown that developmentally distinct cell types employ the same c-*kit* promoter. Nevertheless, it is possible that in other cell lineages c-*kit* may be transcribed from one or more distinct promoters. For example, the human c-*fms* gene has two promoters selectively utilized by monocytes and placental trophoblasts (Roberts *et al.*, 1988). In light of the evolutionary relationship between c-*kit* and c-*fms*, and the conservation of the genomic organization of these genes, it is possible that c-*kit* may also have one or more additional promoters.

B. SCF

As described in Section III, by a combination of post-transcriptional and post-translational mechanisms, the *SCF* gene produces alternative transcripts and protein products whose expression appears to be regulated in a tissue-specific manner. In theory, tissue-specific alternative mRNA splicing could result in the differential expression of soluble as opposed to cell-associated forms of the SCF protein during development and cell differentiation. *SCF* is expressed during development by stromal cells located along the migratory pathway and at the destinations of migrating SCF-positive cells such as melanoblasts and primordial germ cells, suggesting that the primary function of the SCF/SCFR interactions during development may be to direct cell migration. And the spatial and temporal distribution of *SCF* mRNA expression in the tissues of the developing mouse embryo suggests that *SCF* expression may be responsive to regulatory signals generated from migrating c-*kit*-positive cell lineages (Matsui *et al.*, 1990; Keshet *et al.*, 1991). However, very little is known about the mechanism of regulation of *SCF* expression during development or differentiation.

Huang *et al.* (1992) investigated the tissue distribution of the two major SCF transcripts (which they designated KL-1 and KL-2) in mice, and analyzed the generation of soluble SCF from each of the two products in an *in vitro* transient transfection assay. Using an RNase protection assay, Huang *et al.* (1992) determined that the expression of KL-1 and KL-2 appeared to be regulated in a tissue-specific manner. For example, the ratios of KL-1 to KL-2 transcripts in various tissues of a 40-day-old mouse, based on densitometry of blots, were 1.5:1 in spleen, 1:2.6 in testis, and 26:1 in brain. KL-1 was the predominant transcript in *BALB*/c 3T3 fibroblasts, which exhibited barely detectable KL-2, as well as in adult mouse thymus. However, substantial amounts of each transcript were present in placenta (14 days p.c.) and heart.

When production of soluble KL by COS cells transfected with KL-1 or KL-2 expression constructs was measured, both KL-1 and KL-2 released soluble products, but the KL-2-derived product appeared in

the cellular supernatants at later intervals and in lower amounts than did the KL-1-derived product. Moreover, treatment of the transfected COS cells with PMA or the calcium ionophore A23187 enhanced the generation of soluble forms of KL-1 or KL-2 at similar rates. The proteolytic cleavage of either KL-1 or KL-2 transmembrane products occurred at the cell surface, although the specific protease(s) involved remains to be determined (Huang *et al.*, 1992). Although these results have yet to be confirmed *in vivo*, the results suggest that both forms of SCF, including the molecule lacking the exon 6 amino acids (KL-2 or SCF-2), can undergo proteolytic cleavage to produce soluble SCF.

As noted by Huang *et al.* (1992), SCF-dependent function may vary depending on whether the SCFR+ target cell encounters the transmembrane forms of SCF, soluble forms in solution, or soluble forms sequestered among extracellular matrix proteins. Accordingly, the proteolytic release of soluble forms from transmembrane SCF may represent one level of control over SCF function.

Cytokines and other factors produced in the microenvironment of cells that express SCF may also contribute to the regulation of SCF production. Brief exposure to inflammatory cytokines such as TNF-α and IL-1α may down-regulate SCF mRNA levels in human bone marrow stromal cells *in vitro* (D. F. Andrews *et al.*, 1991). This phenomenon presumably might be involved in the suppression of hematopoiesis that is observed in some chronic inflammatory diseases associated with elevated levels of these inflammatory cytokines (Ratajczak *et al.*, 1992b). Moreover, mast cells represent a potential source of both IL-1 and TNFα (Young *et al.*, 1987; Burd *et al.*, 1989; Gordon and Galli, 1990a; Galli, 1993), raising the possibility that, under some circumstances, mast cell cytokine production could down-regulate local production of SCF and thus contribute either to maintaining normal levels of tissue mast cells or to the changes in mast cell numbers that are associated with some perturbations of homeostasis.

In some settings, cytokines may increase SCF production. For example, Aye *et al.* (1992) reported that human umbilical vein endothelial cells express *SCF* and c-*kit* mRNA and that the expression of *SCF* but not c-*kit* mRNA was increased by the addition of either monocyte supernatants, IL-1β, or thrombin to the culture medium. Finally, in a preliminary report, Linenberger *et al.* (1991) found that hydrocortisone moderately increased the level of *SCF* mRNA in bone marrow stromal cells from normal donors as well as from patients with Diamond–Blackfan anemia. However, hydrocortisone treatment had little if any effect on the amount of secreted SCF protein. Further analyses of phenomena such as these, as well as characterization of the

SCF promoter, should shed light on the mechanisms of *SCF* regulation during development and differentiation, or in the setting of pathologic conditions.

VII. Signal Transduction through the SCF Receptor

The SCFR/SCF system not only importantly influences the development of diverse cellular lineages, but does so as a result of its ability to regulate a remarkable spectrum of cellular activities. As described in more detail later, the cellular programs influenced by SCF include adherence, migration, survival, proliferation, differentiation/maturation, and secretion. These diverse responses to SCF may reflect, among other factors, the isoform of the ligand, its density (in the cell-associated forms) or concentration (the soluble form), and its presentation in context with other cell-associated, soluble, or extracellular matrix-associated molecules. Responses also may vary according to the lineage, stage of differentiation/maturation, or functional status of the target cells. Such factors may influence the density and perhaps the isoform of the SCF receptors displayed by the cells, as well as the nature of the additional signaling pathways that may interact with that initiated at the SCFR.

Accordingly, it will be of great interest to examine specific patterns of signal transduction through the SCFR in a spectrum of distinct lineages that express this receptor. To date, however, most analyses of SCFR/SCF signaling have focused on COS cells transfected with natural or chimeric forms of the SCFR, or mast cells. Rottapel *et al.* (1991) reported that SCF induced the SCFR of mast cells to autophosphorylate on tyrosine and to bind phosphatidylinositol 3′-kinase and phospholipase C-γ1, and provided evidence that tyrosine phosphorylation of the SCFR promoted complex formation with PI3′K. This study showed that the SCFR is distinct from CSF-1R by virtue of its ability to bind to PLC-γ1 and is different from the PDGFR in that it apparently cannot bind to the Ras GTPase-activating protein. Rottapel *et al.* (1991) therefore proposed that the distinct affinities of these receptors for their cytoplasmic ligands, together with variation in their pattern of tissue-specific expression, may determine their specific biological effects. Furthermore, they suggested that the variable effects of distinct *W* mutations on different cell lineages might be controlled by the availability of individual substrates within the different lineages. This group also showed that W^{37} and W^{41} mutations, which result in full or partial inactivation of the receptor's tyrosine kinase activity, also

greatly or partially diminish the SCF-induced association of PI3'K and PLC-γ1 with the respective mutant receptors (Reith *et al.*, 1991). Duronio *et al.* (1992) showed that stimulation of mouse hematopoietic or mast cell lines with SCF *in vitro* also resulted in activation of $p21^{ras}$. In accord with the results of Rottapel *et al.* (1991), activation of $p21^{ras}$ in cloned mast cells by SCF or IL-4, IL-5, or GM-CSF was not associated with tyrosine phosphorylation of $p21^{ras}$ GTPase-activating protein (Duronio *et al.*, 1992).

Lev *et al.* (1991) investigated the signal transduction mediated by a chimeric receptor kinase composed of the extracellular ligand-binding domain of the epidermal growth factor (EGF) receptor and the transmembrane and cytoplasmic domains of the SCFR. They demonstrated that on binding EGF, the ligand–receptor complex underwent endocytosis and degradation, and showed that the activated SCFR kinase strongly associated with PI3'K and an 85-kDa phosphoprotein and coupled to PLC-γ1 and the Raf-1 protein kinase, but did not significantly change levels of inositol phosphate. Based on the observed differences in the signaling observed after ligand binding to this chimeric receptor, and that induced via the receptors for CSF-1 or PDGF, Lev *et al.* (1991) also proposed that each receptor in this family is coupled to a specific combination of signal transducers that determines a receptor-specific program of gene expression.

Lev *et al.* (1992a,b,c) then defined the domains of the SCFR that mediate various components of the signal transduction pathway. This work showed that the kinase insert of the receptor mediated coupling with PI3'K and that activation of Raf-1 required an intact C terminus. Moreover, this group showed that a recombinant SCFR kinase insert could not bind PI3'K unless it had been phosphorylated on tyrosine, indicating that tyrosine phosphorylation of the kinase insert was critical to this element of the signal transduction process.

Because many mutant *W* alleles produce SCF receptors that not only have diminished function but confer a dominant negative phenotype in heterozygous mice, it was assumed that activation of the SCFR might be associated with receptor dimerization. Moreover, soluble forms of SCF were shown to form noncovalently linked dimers spontaneously in solution (Huang *et al.*, 1990; Arakawa *et al.*, 1991). Several lines of evidence now have shown that binding of the natural, dimeric form of SCF rapidly induces SCFR dimerization (Blume-Jensen *et al.*, 1991; Lev *et al.*, 1992a). In addition, analysis of cells that coexpressed human and mouse mutant forms of the SCFR indicated that receptor dimerization also can be induced by monovalent binding of the ligand

(Lev *et al.*, 1992a). Accordingly, Lev *et al.* (1992a) have proposed that exposure to SCF may first result in monovalent binding of the ligand to the SCFR, followed by the production of an intermediate receptor dimer bound by one arm of the ligand molecule.

Yarden and his associates have also analyzed the ligand-binding site of the SCFR, using a series of deletion mutants of the ectodomain of the SCFR, chimeric receptors consisting of various proportions of human or mouse sequence, and monoclonal antibodies that interfere with the binding of human SCF to the various receptors (Blechman *et al.*, 1992). This work has determined that human SCF binds primarily to the first two Ig-like domains of the amino-terminal half of the SCFR ectodomain, but that structural determinants distal to this region may also contribute to ligand recognition.

One of the elements involved in both SCF-dependent and CSF-1-dependent signal transduction may be encoded at or regulated by the *mi* locus on mouse chromosome 6 (Silvers, 1979b). Dubreuil *et al.* (1991) showed that tranfection of the CSF-1R (c-*fms*) gene rendered mast cells derived from *W* mutant mice, but not mast cells derived from *mi*/*mi* mice, able to proliferate in response to coculture with fibroblasts. Based on these findings, Dubreuil *et al.* (1991) suggested that binding of ligand to either the SCFR or the CSF-1 receptor activates similar or overlapping signal transduction pathways, and that the *mi* locus may encode an element common to these pathways. More recent data indicate that *mi* also contributes to the regulation of levels of mRNA of both SCFR and protease genes in mast cells (Ebi *et al.*, 1992), and impairs the chemotactic responsiveness of mast cells to a factor in the supernatants of 3T3 cells, which is probably not SCF (Jippo-Kanemoto *et al.*, 1992). Taken together, these findings raise the possibility that *mi* may regulate the expression of several genes, some of which may be involved in the SCFR and CSF-1R signaling pathways.

The binding of extracellular ligands to receptors that have intrinsic tyrosine kinase activity can also induce changes in early response gene expression. For example, the expression of c-fos and c-jun mRNAs can be induced when quiescent fibroblasts are stimulated to enter the cell cycle by platelet-derived growth factor (Greenberg and Ziff, 1984; Kruijer *et al.*, 1984), or when monocytes/macrophages are stimulated by colony stimulating factor-1 (Mufson, 1990; Müller *et al.*, 1985). Because the receptors for PDGF and CSF-1 exhibit structural homology with the c-*kit* receptor, Tsai *et al.* (1993) investigated whether early response genes were induced by the stimulation of the c-*kit* receptors of the mouse bone marrow derived-cultured mast cells. Tsai

et al. (1993) found that SCF-dependent stimulation of the SCF receptors in BMCMCs can lead to increased levels of mRNAs for the c-*fos* gene and for the genes of the *jun* family. These findings thus demonstrated that the induction of these protooncogenes represents one of the immediate responses of at least one cell type, the mast cell, to activation of the SCFR by its ligand.

However, as far as mouse mast cells are concerned, the results of Tsai *et al.* (1993) indicate that one may not be able to establish a direct correlation between patterns of transcription of early response genes per se and subsequent cell proliferation. For example, Tsai *et al.* (1993) found that both SCF (50 ng/ml) and IL-3 (100 pg/ml) exerted strong mitogenic effects on quiescent mouse BMCMCs and, in parallel, induced increased accumulation of early response gene mRNA levels. However, IL-3 at 50 pg/ml also induced significant proliferation of quiescent cells, but produced little or no change in the cells' levels of early response gene mRNAs.

Moreover, BMCMCs stimulated by SCF or by IgE and specific antigen exhibited a strikingly similar pattern of early response gene induction. The results using BMCMCs triggered with IgE and antigen confirm those of Baranes and Razin (1991), who demonstrated a similar pattern of induction of early response genes in Abelson virus-transformed mouse liver-derived mast cells stimulated through the FcεRI. However, the effects of SCF or IgE and antigen on BMCMC proliferation were quite distinct. SCF induced proliferation of BMCMCs. In contrast, Tsai *et al.* (1993) not only confirmed that stimulation of the cells through the FcεRI failed to *induce* proliferation, but found for the first time that such cells exhibited significantly *diminished* proliferation in response to either IL-3 or SCF (73 or 61% reduction versus unstimulated control cells, respectively.) Thus, activation of BMCMCs through the FcεRI, an event that represents the key step in the induction of immunologically specific mast cell function, results in a significant suppression of the cell's ability to proliferate in response to two major growth factors for mouse mast cells, IL-3 and SCF. The mechanism by which FcεRI cross-linking can inhibit mitogen-induced proliferation in BMCMCs is unknown. In light of evidence that stimulation of BMCMCs by IgE and specific antigen down-regulates c-*kit* mRNA levels (Geissler *et al.*, 1990), one could speculate that there are bidirectional interactions between the SCF receptor (and perhaps the IL-3 receptor) and the FcεRI receptor that influence their expression and/or functional activity.

VIII. Biological Consequences of Interactions between the SCF Receptor and Its Ligand

A. Adhesion

The membrane-associated form of SCF can mediate the adhesion of cells that express the SCFR. This point was first established by Flanagan *et al.* (1991), who demonstrated that *in vitro*-derived mouse mast cells adhered to COS cells that had been transfected with the membrane-associated (wild type) but not the soluble (Sl^d) form of SCF. Subsequently, Kaneko *et al.* (1991) and Adachi *et al.* (1992) demonstrated that fibroblasts derived from mice with mutations at *Sl* exhibited a greatly impaired ability to bind *in vitro*-derived mast cells. Adachi *et al.* (1992) also showed that *in vitro*-derived mast cells of *W/W* origin, which express no SCFR on their surface, are markedly impaired in their ability to bind to fibroblasts. In contrast, mast cells derived from *W* mutant mice with markedly diminished (W^v/W^v) or absent (W/W^{42}) SCFR tyrosine kinase activity, but which expressed the SCFR ectodomain on their surface, exhibited no detectable impairment of their ability to adhere to fibroblasts *in vitro* (Adachi *et al.*, 1992). SCF may also promote attachment of mouse primordial germ cells (Matsui *et al.*, 1991). Finally, Avraham *et al.* (1992b) showed that the binding of human megakaryocytes to fibroblasts can be inhibited with a neutralizing antibody to the SCFR.

Taken together, this work indicates that the extracellular domain of the SCFR can mediate the attachment of mouse or human cells to fibroblasts, and that apparently normal levels of attachment can be expressed even by cells bearing SCFR mutant forms that lack any detectable tyrosine kinase activity.

B. Chemotaxis/Haptotaxis

Three of the four most dramatic phenotypic abnormalities in *W* or *Sl* mutant mice, the lack of melanocytes in the skin, the mast cell deficiency, and the sterility, were known to reflect, at least in part, the impaired migration of cells in the melanocyte, mast cell, or germ cell lineage during embryonic development (Russell, 1979; Silvers, 1979a; Kitamura, 1989). With the identification of allelism between *W* and c-*kit*, and between *Sl* and *SCF*, it was reasonable to assume that cells that expressed the SCFR might exhibit a chemotactic response to a concentration gradient of soluble SCF, or that such cells might respond by directed migration along a concentration gradient of insoluble ligand, a process designated haptotaxis.

The skin of Sl/Sl^d mice ordinarily lacks mast cell precursors as well as mature mast cells (Kitamura and Go, 1979; Hayashi *et al.*, 1985). However, we showed that repeated subcutaneous injection of rrSCF^{164} into Sl/Sl^d mice resulted in the appearance of large numbers of dermal mast cells at the injection sites (Zsebo *et al.*, 1990b; Tsai *et al.*, 1991a). Accordingly, this finding must reflect the increased recruitment and/or survival of mast cell precursors at the rrSCF^{164} injection sites.

Blume-Jensen *et al.* (1991) later provided the first direct demonstration that SCFR/SCF interactions could promote chemotaxis; they showed that porcine aortic endothelial cells transfected with wild-type human c-*kit* cDNA expressed cell surface SCFR and exhibited a chemotactic response to concentrations of rhSCF as low as 50 pg/ml. Maximal responses occurred at concentrations of SCF of 5–200 ng/ml, with decreased migration at higher concentrations of the ligand.

Subsequently, Meininger *et al.* (1992) demonstrated that *in vitro*-derived normal mouse mast cells or mouse peritoneal mast cells exhibited chemotactic responsiveness to rrSCF, whereas W^{42}/W^{42} mouse mast cells, which lacked detectable SCFR tyrosine kinase activity, did not. Maximal responses occurred at 1.0 ng/ml of rrSCF. This work indicates that chemotaxis to SCF requires SCFR tyrosine kinase activity, as well as the expression of the extracellular binding domain of the receptor.

C. Survival

It is likely that a major biological function of SCF is to promote the survival of cellular lineages that express the SCFR. Certain SCFR$^+$ cells, such as oocytes, melanocytes, and mast cells, reside in the tissues for long periods of time without undergoing proliferation. And several lines of evidence indicate that SCF can promote cell survival without inducing proliferation. For example, WCB6F$_1$-Sl/Sl^d mice, which lack a membrane-associated form of SCF (Flanagan *et al.*, 1991), cannot support the survival of normal (WCB6F$_1$-+/+) mast cells injected into the dermis (Gordon and Galli, 1990b). In contrast, such mast cells survive in the skin of WBB6F$_1$-W^v/W^v mice (Gordon and Galli, 1990b). These findings indicate that the production of membrane-associated SCF may be required for the survival of mast cells in the dermis of mice, or, at least, that whatever soluble SCF might be produced by Sl/Sl^d mice is not sufficient to maintain the viability of normal mast cells in the skin. Transplantation or embryo fusion studies employing *W* or *Sl* mutant mice also indicate that SCF is required for the survival of melanocytes (Silvers, 1979a) and germ cells (Russell, 1979). Analyses of human hematopoiesis *in vitro* employing *Sl/Sl* stromal cells

transfected to express either the membrane-associated or soluble form of SCF indicate that the membrane-associated form of the molecule maintains hematopoiesis for longer periods than does the soluble form (Toksoz *et al.*, 1992). Membrane-associated SCF also is superior to soluble SCF in promoting mouse primordial germ cell survival *in vitro* (Dolci *et al.*, 1991; Matsui *et al.*, 1991). Nevertheless, *in vitro* studies indicate that soluble SCF also can maintain the survival of mouse primordial germ cells (Dolci *et al.*, 1991; Godin *et al.*, 1991; Matsui *et al.*, 1991), hematopoietic stem cells (I. D. Bernstein *et al.*, 1991; McNiece *et al.*, 1991b; Metcalf and Nicola, 1991; Tsuji *et al.*, 1991a; Cicuttini *et al.*, 1992; Migliaccio *et al.*, 1992; N. Williams *et al.*, 1992), human melanocytes (Funasaka *et al.*, 1992), human mast cells (Irani *et al.*, 1992; Valent *et al.*, 1992; Mitsui *et al.*, 1993), or human NK cell precursors (Uittenbogaart *et al.*, 1992) *in vitro*, and can promote the survival of mouse mast cells *in vitro* or *in vivo* (Zsebo *et al.*, 1990b; Tsai *et al.*, 1991a,b). In addition, infection of *in vitro*-derived W/W^v mast cells with a c-*kit* retroviral expression vector confers on these cells the ability to survive in the peritoneal cavity of W/W^v mice *in vivo* (Alexander *et al.*, 1991).

D. Proliferation

In general, SCF expresses the most dramatic effects on the proliferation of $SCFR^+$ cells when acting in synergy with additional stimuli. Thus, under some experimental conditions, mouse primordial germ cells (PGCs) do not proliferate when exposed to levels of soluble SCF in excess of those required for the *in vitro* survival of these cells (Dolci *et al.*, 1991; Godin *et al.*, 1991). In other experiments, soluble SCF did promote the proliferation of mouse PCGs *in vitro*, but more striking effects on PGC proliferation were observed when SCF was used together with leukemia inhibitory factor (LIF) (Matsui *et al.*, 1991). When four growth factors are present, LIF, basic fibroblast growth factor (bFGF), and both soluble and membrane-associated SCF, PGCs proliferate *in vitro* beyond the time they ordinarily would stop growing *in vivo* and give rise to cells resembling undifferentiated embryonic stem cells, which can contribute to chimeras when injected into host blastocysts (Matsui *et al.*, 1992).

Human melanocytes proliferate *in vitro* when SCF is provided with PMA, but SCF alone has little effect (Funasaka *et al.*, 1992). Similarly, SCF by itself has only modest effects on the development of colonies of hematopoietic cells *in vitro*, but acts in synergy with many other growth factors to promote the production *in vitro* of early and intermediate precursors of erythroid, myeloid, and lymphoid lineages.

The mast cell lineage appears to represent an interesting exception to this pattern, in that the proliferation of immature mouse mast cells or freshly isolated mature mouse peritoneal mast cells can be induced in suspension cultures *in vitro* when natural (Nocka *et al.*, 1990b) or recombinant (Tsai *et al.*, 1991b) forms of SCF are added as the only exogenous cytokine, and infection of *in vitro*-derived *W/W* or W/W^v mast cells with a c-*kit* retroviral expression vector repairs the defective proliferative response of these cells to SCF (Alexander *et al.*, 1991). However, SCF also can act synergistically with other growth factors to promote mast cell proliferation. For example, SCF can act synergistically with IL-3 to promote the *in vitro* proliferation of immature mouse mast cells (S. J. Galli *et al.*, unpublished data) or rat mast cells (Haig *et al.*, 1993) in suspension culture, and can act synergistically with IL-3 and/or IL-4 to promote the proliferation of mouse peritoneal mast cells in semisolid medium (Tsuji *et al.*, 1991b; Takagi *et al.*, 1992). Curiously, contact between immature mast cells and 3T3 fibroblasts derived from Sl/Sl^d mice suppresses the proliferative response of these cells to hematopoietic growth factors (IL-3 and IL-4) *in vitro* (Onoue *et al.*, 1989). Whether this observation reflects the effects of the product of the Sl^d allele, or other factors, remains to be determined.

It is not clear why mast cells should represent the only $SCFR^+$ lineage that can significantly proliferate in response to SCF alone. Under some circumstances, mast cells can produce some of the cytokines, such as IL-3 and IL-4, that act synergistically with SCF in promoting the proliferation of $SCFR^+$ hematopoietic cells and mast cells. This fact, evidence that SCF can induce or up-regulate mast cell mediator production, and the finding that SCF can induce cytokine production by immortalized human megakaryocytic cell lines (Avraham *et al.*, 1992a), raise the possibility that some of the actions of SCF on mast cell survival or proliferation may reflect the agent's ability to influence mast cells to produce cytokines with autocrine effects.

E. Differentiation/Maturation

"Differentiation" may be defined as "specialization; the acquisition or the possession of character or function different from that of the original type," whereas "maturation" can be defined as "the process of achieving full development or growth" (Stedman's Medical Dictionary," 1976). Although most processes of cellular differentiation therefore may be considered as examples of maturation, some examples of maturation (e.g., the storage of increasing amounts of histamine within the cytoplasmic granules of maturing mast cells) may not properly be considered examples of differentiation.

During embryonic development and in postnatal life, the lineages comprising the SCFR$^+$ cells that are defective or reduced in number in *W* or *Sl* mutant mice undergo differentiation/maturation: early lymphohematopoietic cells generate differentiated progenies such as red cells, granulocytes, mast cells, and lymphocytes; melanoblasts develop into melanocytes; and primoridal germ cells give rise to mature gametes. Because SCF importantly influences the migration and, especially, survival of SCFR$^+$ lineages, many of the affected lineages are essentially absent in *W* or *Sl* mutant mice. In such circumstances, effects of SCF or the SCFR on differentiation/maturation may be impossible to discern. However, *in vitro* analyses have shown that SCF primarily maintains hematopoietic cells in a primitive state and that differentiation is the result of a second cytokine signal. For instance, in bone marrow cultures, SCF plus erythropoietin (EPO) will promote primarily erythroid proliferation/differentiation whereas SCF plus G-CSF will result in neutrophil development (Anderson *et al.*, 1990; Nocka *et al.*, 1990b; Zsebo *et al.*, 1990a).

When administered *in vivo*, SCF promoted the appearance of mast cells that share phenotypic characteristics of mature "connective tissue-type" mast cells in both Sl/Sl^d and normal mice, as well as in rats, but not in W/W^v mice (Tsai *et al.*, 1991a). Moreover, when *in vitro*-derived W/W^v mast cells that had been infected with a c-*kit* retroviral expression construct were adoptively transferred into the peritoneal cavity of W/W^v mice, some of these mast cells acquired the ability to bind the fluorescent dye berberine sulfate, a feature of mature peritoneal mast cells (Alexander *et al.*, 1991). Although these studies demonstrated that SCF could promote mast cell maturation *in vivo* (Tsai *et al.*, 1991a) or that adequate SCFR function was necessary for mast cell maturation *in vivo* (Alexander *et al.*, 1991; Tsai *et al.*, 1991a), the results may have reflected effects of SCF/SCFR function on mast cell survival and/or proliferation, rather than on maturation per se.

We therefore determined whether soluble SCF could induce mast cell differentiation/maturation when administered as a single agent *in vitro* (Tsai *et al.*, 1991b). It had been shown by Levi-Schaffer *et al.* (1986) that coculture of immature mouse mast cells on 3T3 fibroblasts could induce the mast cells to acquire a more mature phenotype, including increased histamine content, induction of the ability to synthesize and store substantial amounts of heparin, and alteration of the histochemical characteristics of the cells. Furthermore, several lines of evidence indicated that the ability of fibroblasts to maintain mouse mast cell viability *in vitro* required an interaction between SCF

and the SCFR (Kitamura *et al.*, 1989; Galli, 1990; Galli *et al.*, 1992). We found that populations of immature mast cells derived from mouse bone marrow cells *in vitro* acquired a more mature phenotype after 4 to 6 weeks of suspension culture with rrSCF[164] as the only exogenous cytokine: the histamine content of the cells increased ~30-fold, they acquired the ability to synthesize and store increased amounts of heparin, and they became more mature according to histochemical characteristics (Tsai *et al.*, 1991b). Subsequently, using similar but not identical conditions of culture, Gurish *et al.* (1992) confirmed that soluble SCF can induce bone marrow-derived immature mast cells to store increased amounts of histamine and heparin, and also showed that SCF can induce the cells to express increased levels of mRNA for a protease (MMCP-4) that is expressed by mature peritoneal mast cells.

These studies indicate that SCF can promote mouse mast cell maturation *in vitro* when used as the only exogenous cytokine. And when normal mouse or rat bone marrow cells are maintained under appropriate conditions of suspension culture with SCF as the only exogenous cytokine, the cultures eventually contain many mast cells, although the proportion of mast cells in the populations can vary according to the conditions of culture (Medlock *et al.*, 1990, 1992a; Gurish *et al.*, 1992; Wershil *et al.*, 1992a). These findings indicate that SCF can promote mouse or rat mast cell differentiation *in vitro*. On the other hand, bone marrow-derived mouse mast cells maintained in soluble SCF neither became fully mature, as defined by comparison to freshly isolated mouse peritoneal mast cells (Tsai *et al.*, 1991b; Gurish *et al.*, 1992), nor became as mature as did immature mast cells that had been cocultured with 3T3 fibroblasts (Gurish *et al.*, 1992). Nor did freshly purified peritoneal mast cells maintained in SCF *in vitro* retain fully mature phenotypic characteristics (Tsai *et al.*, 1991b). These findings may indicate that factors in addition to SCF are required for full mast cell maturation. Moreover, certain effects of SCF on mouse mast cell phenotype apparently can be antagonized by other cytokines. For example, bone marrow-derived mast cells maintained in SCF plus IL-3 do not express high levels of MMCP-4 mRNA and have a lower histamine content and more immature histochemical phenotype than do cells maintained in SCF alone (Gurish *et al.*, 1992).

Taken together, these findings indicate that SCF can promote mast cell differentiation/maturation, but that the phenotypic characteristics of this cell type are regulated by a complex interplay of SCF and other cytokines. On the other hand, in other SCFR$^+$ lineages, SCF appears to represent primarily a survival and/or proliferation factor rather than a differentiation/maturation factor.

F. Activation or Modulation of Secretion

The cellular lineages that are the most dependent on SCF and SCFR for their survival are essentially absent in the most severely affected *W* or *Sl* mutant mice. Accordingly, analyses of the phenotypic abnormalities of these mutants have provided little or no insight into whether SCF/SCFR interactions could influence the secretory function of $SCFR^+$ cells. However, W/W^v cutaneous mast cells can be generated *in vivo* in W/W^v mice whose skin is repeatedly treated with PMA (Gordon and Galli, 1990b). Studies employing these mice, and W/W^v mice containing adoptively transferred populations of normal ($WBB6F_1 - +/+$) mast cells, showed that SCF can induce SCFR-dependent mast cell activation and mast cell-dependent inflammation *in vivo* (Wershil *et al.*, 1992b). This represents the first demonstration that SCF can activate secretion by stimulating cells through the SCFR. SCF also can induce mediator release from purified human skin mast cells (Columbo *et al.*, 1992) and from purified mouse peritoneal mast cells (Coleman *et al.*, 1993) *in vitro*.

In vitro studies with human lung (Bischoff and Dahinden, 1992) or skin (Columbo *et al.*, 1992) mast cells or with purified mouse peritoneal mast cells (Coleman *et al.*, 1993) also showed that soluble SCF can enhance the level of mast cell secretion and mediator release observed in cells stimulated through the FcεRI. Indeed, this effect of SCF was observed at concentrations of the cytokine that were substantially lower than those required to induce secretion directly.

This work shows that SCF can regulate the secretory activity of mast cell, as well as influence their development. It is not yet clear to what extent SCF also can influence the functional activity of other lineages that express the SCFR. However, recent reports indicate that SCF may augment the cytotoxic activity of certain lymphoid effector cells (Uittenbogaart *et al.*, 1992) and can induce cytokine production by immortalized human megakaryocytic cell lines (Avraham *et al.*, 1992a).

IX. Lymphohematopoiesis

A. Hematopoiesis

In broad outline, hematopoiesis is characterized by the sustained self-renewal of a population of hematopoietic stem cells and by the regulated differentiation from this stem cell pool of various lineages with specialized function, such as red cells, megakaryocytes, granulocytes, monocytes, and mast cells. Many of the most differentiated products of hematopoiesis, such as red cells, platelets, and mature

granulocytes, have both a relatively short half-life and no proliferative ability. Accordingly, throughout the life of the organism these populations must be renewed continuously by differentiation from more primitive precursors.

Much of the regulation of hematopoiesis is accomplished by a multitude of hematopoietic growth factors that influence the survival, proliferation, and/or differentiation of multipontent stem cells or their more differentiated descendants (Quesenberry, 1990; Ikuta *et al.*, 1991). Although many of these growth factors have significant biological activity when present in soluble forms, the earliest stages of hematopoiesis are importantly regulated by contact between hematopoietic cells and stromal cells, such as those in the bone marrow (Quesenberry, 1990), or by hematopoietic factors that are presented in the context of extracellular matrix components (Gordon, 1991). In general, the earlier in hematopoiesis a particular population of hematopoietic cells is positioned, the more difficult it is to maintain such cells in liquid culture in the presence of soluble growth factors alone (Quesenberry, 1990). In addition, stem cells and other early hematopoietic cells represent a very small fraction of the cells present in the bone marrow and other sites of hematopoiesis (Quesenberry, 1990; Ikuta *et al.*, 1991). For these reasons, it has been difficult to isolate and characterize the earliest hematopoietic stem cells. Indeed, such cells are often operationally defined as a population that can exhibit both self-renewal and long-term multilineage reconstitution of lymphohematopoiesis *in vivo*, or identified as residing within a potentially heterogeneous population of cells that can be isolated based on positive or negative selection for a constellation of cell surface structures (Spangrude, 1989; Visser and Van Bekkum, 1990; Ikuta *et al.*, 1991, 1992).

Several approaches have been used to elucidate the role of specific growth factors in hematopoietic stem cell biology and hematopoiesis. In the case of SCF and its receptor, the oldest data were derived from analysis of the phenotypic abnormalities expressed by *W* or *Sl* mutant mice. For example, Chui and Russell (1974) showed that the yolk sac and fetal liver of Sl/Sl^d embryos contained reduced but clearly detectable numbers of pluripotent hematopoietic stem cells (CFU-S). In a more recent study, Ikuta and Weissman (1992) reported that the liver of *Sl/Sl* fetuses contained 30–40% of the hematopoietic stem cells present in the livers of the corresponding +/+ fetuses. Because the *Sl* mutation represents a complete deletion of all SCF coding sequences (see Section III), these findings indicate that embryos lacking any SCF production can nevertheless generate hematopoietic stem cells. Finally, Kodama *et al.* (1992) provided evidence for a stromal cell-

dependent but SCFR/SCF-independent mechanisms for supporting long-term mouse hematopoiesis *in vitro*.

On the other hand, several lines of evidence indicate that hematopoietic stem cells express the SCFR and that SCF/SCFR signaling is necessary for normal hematopoiesis. For example, mice homozygous for the *W* allele, which now is known to encode an SCFR lacking the transmembrane component and which is not expressed on the cell surface (see Section V,A), die perinatally of a severe anemia (Russell, 1979). In contrast, mutants with at least one *W* allele that can express limited SCFR function, such as W^v, have a less severe anemia and exhibit an essentially normal life span (Russell, 1979). Moreover, Ogawa *et al.* (1991) showed that treatment of adult normal mice with the ACK-2 anti-SCFR monoclonal antibody resulted in the virtual disappearance of hematopoietic progenitor cells from the bone marrow and also eventually resulted in the absence of mature myeloid or red cells in the bone marrow. In all, ~8% of total adult mouse bone marrow cells expressed the SCFR, of which about half did not coexpress lineage markers such as Mac-1, Gr-1, TER-119, or B220. And in the study by Ikuta and Weissman (1992), the hematopoietic stem cells present in the fetal livers of *Sl/Sl* embryos also expressed the SCFR. Both Okada *et al.* (1991) and Ikuta and Weissman (1992) reported that only the $SCFR^+$ and lineage $marker^-$ (lin^-) fraction of adult bone marrow cells could reconstitute the lymphohematopoietic system of irradiated mice. These studies indicate that expression of the SCFR in the absence of the significant expression of lineage-specific markers represents a phenotypic characteristic of mouse stem cells capable of giving rise to all lymphohematopoietic lineages.

Taken together, these findings indicate that the development and survival of mouse hematopoietic stem cells can occur in the absence of interactions between SCF and its receptor, but that SCF may augment the numbers and/or the anatomical distribution of such stem cells, and that SCF/SCFR interactions are required for normal hematopoiesis. The observation that *Sl/SCF* mutant mice exhibit abnormal hematopoietic responses to acute injury, such as irradiation, indicates that another major role of SCF may be to contribute to hematopoiesis during states of hematopoietic stress (Russell, 1979). In accord with this hypothesis, Hunt *et al.* (1992) have reported evidence that SCF contributes to the rebound thrombocytosis that follows 5-fluorouracil treatment in mice.

Since the isolation of cDNA for SCF and the generation of recombinant forms of this growth factor, a large number of studies have evaluated the effects of SCF in various *in vitro* assays of hematopoiesis or

lymphopoiesis or have assessed the effects of the growth factor on lymphohematopoiesis *in vivo*. Several major themes have emerged from such work. First, SCF can promote the survival of primitive stem cells *in vitro*, but requires other factors to induce the proliferation and self-renewal of these cells (I. D. Bernstein *et al.*, 1991; McNiece *et al.*, 1991a; Metcalf and Nicola, 1991; Tsuji *et al.*, 1991a; Cicuttini *et al.*, 1992; Migliaccio *et al.*, 1992; N. Williams *et al.*, 1992; Fleming *et al.*, 1993). For example, SCF interacts synergistically with IL-3, IL-6, and G-CSF to enhance mouse blast cell colony and multilineage colony formation by shortening the dormancy period (G_0) of the early progenitors (Tsuji *et al.*, 1991a). SCF also acts synergistically with IL-11 (Tsuji *et al.*, 1992) but not IL-4 (Tsuji *et al.*, 1991a) in augmenting the proliferation of early progenitors in mouse bone marrow cells. Whereas the synergistic interactions between SCF and other growth factors are well described, SCF may also express synergistic effects with extracellular matrix proteins (Long *et al.*, 1992). Finally, McNiece *et al.* (1992) showed that TGF-β can strikingly inhibit the synergistic effects of SCF and other growth factors on *in vitro* colony formation by mouse or human hematopoietic progenitor cells, probably through direct effects on the hematopoietic progenitor cells. This represents one example of how some cytokines might antagonize SCF-dependent augmentation of hematopoiesis.

Second, by transfecting various forms of human SCF into immortalized stromal cell lines derived from the livers of *Sl/Sl* embryos, Toksoz *et al.* (1992) showed that either soluble SCF or membrane-associated SCF can increase the numbers of human hematopoietic progenitor cells present in these coculture systems. However, hematopoiesis was supported for much longer periods *in vitro* in cultures in which the stromal cell layer expressed membrane-associated SCF. This finding, when taken together with the occurrence of defects in hematopoiesis in Sl/Sl^d mice, which do not express a membrane-associated form of SCF, suggests that normal hematopoiesis may require expression of a membrane-associated form of SCF within the hematopoietic microenvironment. On the other hand, it is not yet clear what levels of soluble SCF actually are produced by Sl/Sl^d mice, as opposed to cells transfected with Sl^d cDNA.

Third, while soluble SCF has only modest effects on hematopoietic progenitors when used as a single exogenous cytokine *in vitro*, it can expand and/or change the distribution of hematopoietic progenitor cells when administered as a single agent *in vivo*. Thus, when administered to mice (Molineux *et al.*, 1992; Bodine *et al.*, 1992; Fleming *et al.*, 1993) or baboons (Andrews *et al.*, 1992) *in vivo* as the only exogenous

cytokine, SCF can promote striking increases in the numbers of primitive hematopoietic progenitors in the bone marrow or spleen and in the peripheral blood. In both baboons (Andrews *et al.*, 1992) and mice (Fleming *et al.*, 1993), the peripheral blood mononuclear cells of SCF-treated animals contain greatly increased numbers of cells that confer hematopoietic engraftment and protection on lethally irradiated recipients. Moreover, in mice, Fleming *et al.* (1993) demonstrated that the enhanced radioprotection was conferred by peripheral blood mononuclear cells that could provide long-term, donor-derived multilineage lymphohematopoiesis. In rats, SCF can act synergistically with G-CSF or GM-CSF to augment granulopoiesis *in vivo* (Ulich *et al.*, 1991b), indicating that some of the synergistic effects demonstrated by SCF and other hematopoietic factors *in vitro* may also occur *in vivo*.

Fourth, with the exception of the mast cell (see later), as cells within specific hematopoietic lineages differentiate, they appear to exhibit diminished expression of the SCFR and, in parallel, reduced responsiveness to SCF (Papayannopoulou *et al.*, 1991). In this context, specific lineage commitment is determined by the constellation of other hematopoietic cytokines that are presented together with SCF, as well as by other factors. For example, liquid culture of normal mouse bone marrow cells with SCF can produce predominantly mast cells (Medlock *et al.*, 1990; Wershil *et al.*, 1992a), whereas in semisolid agar cultures SCF promotes formation of mixed colonies containing granulocytes, macrophages, and megakaryocytes (Metcalf and Nicola, 1991). Many studies now have shown that SCF acts in synergy with other growth factors to promote amplification of progenitor cells committed to specific lineages. For example, in both mouse and human bone marrow cultures, SCF plus erythropoietin expands populations of primitive erythroid progenitors (Dai *et al.*, 1991; McNiece *et al.*, 1991a). In various *in vitro* systems, SCF can act synergistically with several different growth factors, including EPO, IL-3, IL-6, GM-CSF, G-CSF, and IL-7, to promote the development of lymphohematopoietic lineages whose differentiation is governed not by SCF, but by the properties of the additional growth factors (R. G. Andrews *et al.*, 1991; Briddell *et al.*, 1991; Broxmeyer *et al.*, 1991a,b; Carow *et al.*, 1991; Lowry *et al.*, 1991; McNiece *et al.*, 1991a,b; Migliaccio *et al.*, 1991).

Finally, SCF treatment can increase numbers of mature leukocytes *in vivo*, but these effects are quantitatively modest when compared to effects on primitive hematopoietic progenitors. For example, when administered to normal mice *in vivo*, SCF modestly increased (by ~2-fold) numbers of peripheral blood neutrophils but markedly increased numbers of IL-3-responsive progenitor cells and CFU-S in the

bone marrow and spleen (Molineux *et al.*, 1992). When administered to baboons *in vivo*, SCF had even more striking effects, producing significant increases (of up to 100-fold) in hematopoietic progenitor cells in the bone marrow and blood, and also increasing peripheral blood red cells, neutrophils, eosinophils, basophils, monocytes, and lymphocytes, but not platelets (R. G. Andrews *et al.*, 1991, 1992). Many, if not all, of these effects are reversible, with values returning to baseline levels within days of discontinuation of SCF treatment (R. G. Andrews *et al.*, 1991, 1992). These findings illustrate that, when given in appropriate amounts, soluble forms of SCF can act as a multilineage hematopoietic growth factor in nonhuman primates *in vivo* and can promote both the expansion of progenitor cell populations in the bone marrow and the appearance of markedly elevated numbers of these cells in the peripheral blood. Of course, many of the effects of SCF *in vivo* may reflect, at least in part, its ability to act synergistically with other, endogenous growth factors. Early results from phase I clinical trials indicate that SCF can also produce substantial increases in levels of hematopoietic progenitor cells in the peripheral blood of humans (Demetri *et al.*, 1993a,b; Kurtzberg *et al.*, 1993; Orazi *et al.*, 1993; Tong *et al.*, 1993).

B. Lymphocytes and NK Cells

Analyses of *W* or *Sl* mutant mice have revealed interesting parallels between the effects of SCF and its receptor on myeloid and lymphoid lineages. In general, the numbers of mature granulocytes or lymphocytes in the peripheral blood of *W* or *Sl* mutant mice are normal, but the animals exhibit diminished numbers of the corresponding progenitor cells (Russell, 1979). And as in the case of myeloid cell development in the bone marrow, early stages of T cell development in the thymus or B cell development in the bone marrow are importantly regulated by cell–cell interactions and growth factors present in the relevant stromal microenvironment (Kincaide *et al.*, 1989; Ikuta *et al.*, 1992). Several lines of evidence now indicate that SCF can influence the development of T cells, B cells, and NK cells, but that SCF-independent mechanisms can compensate for the lack of SCF in early stages of lymphoid cell development and that mature lymphocytes exhibit little responsiveness to SCF.

T cells are derived from bone marrow stem cells that migrate to the thymus; after maturation within the thymus, mature T cells enter the peripheral circulation (Palacios and Pelkonen, 1988). The number of mature lymphocytes in the blood of Sl/Sl^d mice is normal (Russell, 1979); however, the cellularity of the thymi of 12-week-old Sl/Sl^d mice

is reduced by approximately 50% when compared to that of the congenic +/+ mice (Medlock *et al.*, 1992b). SCF transcripts have been observed in a number of thymic stromal cell lines (D. E. Williams *et al.*, 1992), and SCF administration *in vivo* can increase the absolute number of cells that migrate to and colonize the thymus in irradiated rats (Medlock *et al.*, 1992c). On the other hand, mature human peripheral blood lymphocytes express little or no SCF receptor (Papayannopoulou *et al.*, 1991). In addition, SCF had no detectable effect on the proliferation of mouse peripheral blood T cells tested either in the presence of or absence of mitogens or other lymphokines (D. E. Williams *et al.*, 1992). Although SCF has little or no effect on mature peripheral blood T cells, SCF is a potent synergistic factor with IL-2 or IL-7 in promoting the proliferation of $CD4^-/CD8^-$ adult mouse thymocytes (D. E. Williams *et al.*, 1992). Lobe submersion culture studies of day 13 fetal thymus lobes demonstrated a significant synergistic effect of SCF on IL-7-mediated expansion of these thymocytes as well. However, the SCF-mediated expansion of these cells occurred without apparent differentiation, since the cells retained the $CD3^-/CD4^-/CD8^-Thy1^+$ phenotype throughout the experiment.

Studies of thymocytes obtained from normal human thymus specimens indicate that SCF increases the viability of human thymocytes in culture, particularly during periods of culture in excess of 24 days (Uittenbogaart *et al.*, submitted for publication). Most of the surviving cells in cultures with SCF were small thymocytes that dimly expressed CD3, CD4, CD5, CD7, and CD8 antigens. However, a subset of the cells exhibited increased size and granularity and did not express detectable CD3, CD4, CD8, CD14, CD16, CD19, CD34, or CD56. When human thymocytes were cultured with SCF in combination with other cytokines such as IL-4 or IL-7, the cells exhibited the phenotypic changes that would be expected based on exposure to the second cytokine. Thus, the predominant cells cultured with IL-4 or SCF plus IL-4 were large, bright $CD3^+CD45RA^+$ lymphocytes, a subset of which expressed TCR γ/δ. Similar results were obtained with IL-7 plus SCF except that very few TCR γ/δ cells appeared. When thymocytes were cultured in the presence of SCF and IL-2, there was a 2- to 10-fold increase in the number of large granular cells expressing the CD56 antigen as compared to thymocytes cultured with either cytokine alone. In addition to an increase in the percentage of $CD56^+$ cells, indicative of an NK/LAK phenotype, there was a corresponding increase in the cytotoxic activity of these cells against either NK-sensitive or NK-resistant targets. SCF has also been shown to augment IL-2-induced proliferation of NK cells from human peripheral blood

(Matos *et al.*, 1992). These results suggest that SCF may promote the survival or development of NK/LAK precursors in the thymus and may act synergistically with IL-2 in augmenting the cytotoxic effector function of these cells.

The effects of SCF on the expansion of NK precursors from thymocytes is intriguing in light of recent evidence indicating that NK cells may contribute to the anemia of Sl/Sl^d mice (Pantel *et al.*, 1991). NK cells may exert either positive or negative effects on aspects of hematopoiesis *in vitro* (Pistoia *et al.*, 1989) or *in vivo* (Pantel *et al.*, 1991). NK cells can elaborate a number of cytokines, some of which, such as GM-CSF and IL-3, exert a positive influence on hematopoiesis (Pistoia *et al.*, 1989) and others of which, such as TNFα, can have a negative effect on myeloid development. Administration of an antibody against NK cells to Sl/Sl^d mice resulted in an increase in bone marrow cellularity and erythroid and myeloid progenitor cell numbers (Pantel *et al.*, 1991). By contrast, the megakaryocytic lineage was suppressed by the administration of anti-NK cell antibodies. Although the complex control mechanisms underlying these effects remain to be elucidated, the results suggest that NK cell activity may contribute to some of the hematological abnormalities expressed by Sl/Sl^d mice.

In adults, primary B cell production from lymphohematopoietic stem cells takes place in the bone marrow (Tidmarsh *et al.*, 1989; Kincaide *et al.*, 1989; Ikuta *et al.*, 1992). The earliest B lineage cell surface marker detected on murine cells is B220 (Hardy *et al.*, 1991). Immature pre-B cells have rearranged diversity (D) and joining (J) region segments on a least one allele of the Ig heavy (H) chain locus and are $B220^+$. Immature pre-B cells differentiate into pre-B cells on variable (V)–DJ joining and expression of cytoplasmic μ followed by expression of κ or λ light chain proteins. Pro-B cells are the precursors to pre-B cells and are defined as B lineage-restricted cells that retain Ig genes in the germ-line configuration yet have the capacity to differentiate into mature B cells. Once pre-B cells acquire the presence of the B220 antigen, they become responsive to IL-7. However, $B220^-$ cells are not responsive to IL-7 in the absence of stromal layers (Hardy *et al.*, 1991; Hayashi *et al.*, 1990).

Adult *W* or *Sl* mutant mice have normal numbers of B cells in the spleen and bone marrow, but embryonic W/W^v and W^x/W^x mice have reduced absolute numbers of pre-B cells in the fetal liver and these cells are less capable of generating splenic B cells in irradiated recipients (Landreth *et al.*, 1984). The effects of SCF on B cell lymphopoiesis has been analyzed in primary bone marrow-derived pre-B cells as well as in pre-B cell lines. McNiece *et al.* (1991b) studied primary bone

marrow cells from mice separated by immunomagnetic bead selection for $B220^+$ and $B220^-$ populations. As expected, recombinant IL-7 supported the proliferation of B cells only from the $B220^+$ population. SCF alone supported the proliferation of small myeloid colonies from the $B220^-$ populations. However, when both factors were combined, there was a substantial increase in the size of colonies produced from either population, and all of the progeny were $B220^+$. Similar experiments were performed by Billips *et al.* (1992), who used three cycles of panning, and Funk *et al.* (1993), who used cell-sorting techniques, to purify the $B220^+$ and $B220^-$ populations from normal mouse bone marrow cells. When these highly purified cells were used, no stimulation of B lymphopoiesis by IL-7 plus SCF from $B220^-$ pre-B cells was observed.

Taken together, these experiments with soluble SCF indicated that the cytokine can act synergistically with IL-7 to promote expansion of $B220^+$ cells, but left unresolved the issue of whether SCF can influence $B220^-$ cells. However, several lines of evidence suggest that direct cell–cell interactions are required for the maintenance and growth of $B220^-$ precursors (Hayashi *et al.*, 1990). Developing B lymphocytes express the VLA-4 integrin that interacts with what appears to be a VCAM-1 ligand on stromal cells. In addition, hyaluronate present on stromal cells has been implicated in mediating stromal cell–B lymphocyte interactions. Rolink *et al.* (1991) examined the role of SCF/SCF receptor interactions between pre-B cell lines and stromal cells by utilizing the ACK-2 neutralizing antibody to the SCF receptor. Addition of the ACK-2 antibody to the stromal cell cultures inhibited the growth of these pre-B cell lines, which require both contact with stromal cells and the costimulatory activity of IL-7 for continuous proliferation. This result suggests that SCF expressed by stromal cells may represent one of the stimulatory signals needed for the proliferation of these cloned pre-B cell lines. However, normal pro-B cells derived from murine bone marrow will grow on the S17 stromal cell line (Saffran *et al.*, 1992) but will not grow in response to SCF plus IL-7, even though these cells express receptors for both cytokines (Faust *et al.*, 1993). Moreover, these cells proliferate on murine stromal lines derived from *Sl/Sl* embryos, which have a deletion in the SCF gene (Zsebo *et al.*, 1990b). Hence, SCF is not an obligate growth factor for the proliferation of mouse pro-B cells.

Taken together, these data indicate that SCF is a potent comitogen for normal $B220^+$ pre-B cells and certain pre-B cell lines, but cannot replace the stromal signal necessary for the proliferation of normal pro-B cells.

X. Mast Cell Biology

A. Mast Cell Development

Mast cells are distributed throughout essentially all vascularized mammalian tissues, where they occur in close proximity to blood vessels, nerves, epithelia, and smooth muscle. They can be particularly abundant in proximity to surfaces exposed to the environment, such as in the skin and in the respiratory, gastrointestinal, and urogenital tracts. On appropriate stimulation, they can produce a diverse array of biologically active mediators, including biogenic amines, cytokines, products of arachidonic acid oxidation through either the cyclooxygenase or lipoxygenase pathways, neutral proteases, and proteoglycans (Galli, 1990, 1993). They express the FcεRI on their surface, and thereby represent an important effector cell population in IgE-dependent responses, including those associated with host reactions to parasites or allergic diseases. However, they also can be activated for mediator secretion by products of complement activation, certain neuropeptides, insect or reptile venoms, products of leukocytes, and a wide variety of other agents (Galli, 1990, 1993). This broad pattern of reactivity, taken together with other lines of evidence, has suggested that mast cells contribute to a wide variety of biological responses in addition to IgE-dependent immune reactions (Galli, 1990, 1993).

Even though abnormalities in mast cell development were among the last major phenotypic problems to be identified in *W* or *Sl* mutant mice, analyses of this lineage have provided the clearest and most complete picture of the spectrum of biological processes that can be importantly influenced by interactions between SCF and its receptor. In a sense this is fitting, because analyses employing *W* or *Sl* mutant mice, most of them performed before the genes affected by these mutations had been identified, have produced the clearest understanding of the development and function of the mast cell (reviewed in Kitamura, 1989; Galli *et al.*, 1992). To cite one early and important example, transplantation studies employing W/W^v and congenic normal or semisyngeneic "beige" mice provided the most convincing demonstration that tissue mast cells are derived from hematopoietic precursors (Kitamura *et al.*, 1978).

Work employing *W* or *Sl* mutant mice, most of it performed by Kitamura and his associates, has shown that mast cell precursors reside among hematopoietic cells in the fetal yolk sac and liver, and then in the fetal and adult bone marrow and spleen (Kitamura, 1989). Cells capable of becoming mast cells circulate in the peripheral blood as a

minor population which cannot be identified based on morphological characteristics such as prominent cytoplasmic granules. These mast cell precursors enter the peripheral vascularized tissues and serosal cavities, where they differentiate/mature into mast cell populations that can be identified based on their characteristic morphological, biochemical, or functional properties. Although all mature mouse mast cells share certain phenotypic characteristics, such as the expression of large numbers of FcεRI in the plasma membrane and the storage of histamine and serotonin in the cytoplasmic granules, certain other aspects of phenotype, such as the types of cytoplasmic granule-associated serine proteinases and proteoglycans, can vary. Moreover, much of this phenotypic variation, often designated "mast cell heterogeneity," appears to be regulated by factors present in the anatomical microenvironments within which mast cell populations reside (Galli, 1987, 1990; Kitamura, 1989; Stevens and Austen, 1989; Galli *et al.*, 1992). The factors that influence mast cell heterogeneity have been of interest because this phenomenon represents a model for investigating the regulation of gene expression during cellular maturation/differentiation, but also because factors that influence important aspects of mast cell phenotype and function can determine the nature of the contributions of the cell to health and disease (Galli, 1987, 1990; Kitamura, 1989; Stevens and Austen, 1989; Galli *et al.*, 1992).

In addition to expressing variation in important aspects of phenotype, at least some of the apparently mature mast cells residing in peripheral tissues or serosal cavities retain the ability to reenter the cell cycle and to proliferate (Galli *et al.*, 1984, 1992; Kitamura, 1989). Finally, even clonal populations of mouse mast cells can exhibit significant and reversible alterations in multiple aspects of their phenotype *in vitro* or *in vivo* (Kanakura *et al.*, 1988). In short, the mast cell lineage is quite plastic in phenotype, as well as in proliferative capacity (Galli, 1990, 1993). Clearly, such plasticity may permit this lineage to respond to microenvironmental changes during development, tissue remodeling, immune responses, or disease with adpative alterations in its numbers, mediator content, and functional properties.

Although some details of mast cell development and phenotype differ in mice and humans (sometimes significantly), the major themes are the same. Thus, the circulating precursors are not recognizable as mast cells morphologically, maturation/differentiation takes place in the peripheral tissues, mast cell subpopulations exhibit variations in multiple aspects of phenotype, and at least some apparently mature mast cells in the peripheral tissues can undergo proliferation (re-

viewed in Galli *et al.*, 1984). Accordingly, many findings derived from studies of mouse mast cells may have substantial relevance to the biology of mast cells in humans.

The fact that the tissues of *W* or *Sl* mutant mice virtually lacked mast cells did not escape the notice of those groups that were seeking to identify the ligand for the receptor encoded by c-*kit*. Using various combinations of mast cells or their precursors and fibroblasts derived from *W* and *Sl* mutant mice, both Kitamura and Fujita *et al.* (Kitamura *et al.*, 1989; Fujita *et al.*, 1989; Kitamura and Fujita, 1989) and Jarboe and Huff (1989) reported strong biological evidence that fibroblasts can produce cell-associated (Kitamura *et al.*, 1989; Fujita *et al.*, 1989; Kitamura and Fujita, 1989) and soluble (Jarboe and Huff, 1989) forms of the c-kit ligand. And each of the three groups that succeeded in cloning the c-kit ligand used mast cell proliferation *in vitro* either as their primary bioassay for the growth factor or in the characterization of the spectrum of factor bioactivity (see Section III,A). However, the phenotypic abnormalities expressed by *W* or *Sl* mutant mice suggested that interactions between the SCFR and SCF might regulate the migration and/or maturation of the cells in the affected lineages, as well as their proliferation (Kitamura, 1989). Studies of the effects of SCF on mast cell development in mice and rats indicate that interactions between the SCFR and SCF actually do influence all of these aspects of mast cell biology.

For example, repeated injection of soluble recombinant rat SCF[164] into the skin of Sl/Sl^d mice resulted in the appearance of large numbers of mast cells at the injection site (Table II) (Zsebo *et al.*, 1990a; Tsai *et al.*, 1991a). Because the skin of S/Sl^d mice ordinarily lacks mast cell precursors, as well as mature mast cells (Kitamura and Go, 1979; Hayashi *et al.*, 1985), the recruitment and/or survival of circulating mast cell precursors must have been increased at sites injected with rrSCF[164]. Yet many of the mast cells that developed in the skin of rrSCF[164]-treated Sl/Sl^d mice, like the mature mast cells in the dermis of normal mice, exhibited reactivity with safranin and the heparin-binding fluorescent dye berberine sulfate (Tsai *et al.*, 1991a), as well as a high rate of incorporation of bromodeoxyuridine (Table II) (Tsai *et al.*, 1991a).

These findings indicate that the development of large numbers of dermal mast cells in Sl/Sl^d mice treated with rrSCF[164] reflects not only the recruitment and maturation of mast cell precursors, which may reflect the chemotaxis or haptotaxis of these cells along an SCF gradient (Flanagan and Leder, 1990; Flanagan *et al.*, 1991; Meininger *et al.*, 1992), but also proliferation of the mast cells that differentiated/

TABLE II
c-*kit*-DEPENDENT STIMULATION OF PROLIFERATION OF MOUSE DERMAL MAST CELLS *in Vivo* BY rrSCF[164][a]

Mouse genotype	rrSCF164 (μg/kg/day)	Hematocrit (%)		Mast cells (No./mm^2 of dermis)	BrdU (percentage of mast cells)
		Before treatment	After treatment		
WBB6F$_1$-+/+	100	48 ± 1.3	50 ± 0.7	4710 ± 1190[b]	16 ± 2.2[b]
	30	50 ± 0.8	51 ± 0.3	454 ± 155[b]	13 ± 2.7[b]
	0	51 ± 0.6	51 ± 0.5	31 ± 2	3.3 ± 1.2
WBB6F$_1$-*W*/*W*^{v}	100	39 ± 0.8	36 ± 3.4	0	—
	30	40 ± 0.7	40 ± 2.5	0	—
	0	40 ± 0.6	39 ± 0.2	0	—
WCB6F$_1$-+/+	30	46 ± 0.8	44 ± 2.7	122 ± 17[b]	36 ± 3.5[b]
	0	47 ± 0.3	44 ± 0.4	29 ± 5.	4.9 ± 0.7
WCB6F$_1$-*Sl*/*Sl*^{d}	30	30 ± 1.1	38 ± 1.7[b]	61 ± 24[b]	29 ± 5.7
	0	30 ± 0.3	33 ± 0.6	0	—

[a] Mice were killed 24 hours after the last of 21 daily subcutaneous injections of rrSCF[164] or vehicle for assessment of the number of mast cells/mm^2 of dermis at the injection site and for quantification of the percentage of these mast cells that were proliferating, based on nuclear incorporation of bromodeoxyuridine (BrdU). All results are expressed as mean ± SEM (n = 5–7). Reproduced from Tsai *et al.* (1991a) with permission.

[b] $p < 0.001$ versus value for mice treated with 0 μg/kg/day by Student's t test (two-tailed).

matured at these sites (Tsai *et al.*, 1991a). *In vitro* studies with purified endogenous c-kit ligand (Nocka *et al.*, 1990b) or rrSCF[164] (Tsai *et al.*, 1991b) also indicated that SCF not only can drive the proliferation of phenotypically mature, as well as immature, mouse and mast cells (Nocka *et al.*, 1990b; Tsai *et al.*, 1991b), but in addition can promote mast cell maturation (Tsai *et al.*, 1991b).

In rats, intravenous administration of rrSCF[164] induces a remarkable systemic mast cell hyperplasia (Tsai *et al.*, 1991a; Ulich *et al.*, 1991a). Notably, assessment of the cellular histochemical characteristics and cytoplasmic granule protease content indicates that the expanded mast cell populations present in different organs of rrSCF[164]-treated rats exhibit phenotypic characteristics that are, for the most part, appropriate for that anatomical site (Tsai *et al.*, 1991a). Thus, mast cells with characteristics of "connective tissue-type" mast cells (CTMCs), such as prominent expression of the proteinase rat mast cell proteinase I (RMCPI), are increased in number in the dermis and other sites that ordinarily contain such cells, whereas increased numbers of mast cells with characteristics of "mucosal" mast cells (MMCs), such as prominent expression of RMCPII, appear in the mucosae of the stomach and ileum, sites that ordinarily contain MMCs (Tsai *et al.*, 1991a).

One of the possible explanations for the findings in rats injected with

rrSCF^{164} is that the specific programs of gene expression, and the phenotypic characteristics of those mast cells induced to develop in the presence of exogenous SCF in different anatomical sites *in vivo*, are regulated both by SCF and by the pattern of expression at these sites of other factors capable of influencing mast cell development. Several lines of evidence indicate that other cytokines represent an important component of such microenvironmental regulation of the phenotype of the mast cells that develop in SCF-treated animals. In mice, IL-3 can promote the *in vitro* development of immature mast cells that share certain features of MMCs and can induce the development of MMCs *in vivo* (reviewed in Kitamura, 1989; Galli, 1990; Galli *et al.*, 1992). An the maintenance of mouse peritoneal mast cells *in vitro* in media containing IL-3 and IL-4 can induce this population of proliferate and gradually to lose phenotypic characteristics of mature CTMCs in favor of those of immature mast cells (Kanakura *et al.*, 1988). Finally, Gurish *et al.* (1992) recently demonstrated that the ability of SCF to induce immature mouse mast cells to acquire phenotypic characteristics of CTMCs *in vitro* is suppressed when the cells are treated simultaneously with SCF and IL-3.

Both the studies of the effects of rrSCF^{164} on rat mast cell development *in vivo* (Tsai *et al.*, 1991a) and analyses of the effects of SCF and IL-3 on mouse mast cell phenotype *in vitro* (Gurish *et al.*, 1992; Takagi *et al.*, 1992) indicate that SCF does not invariably promote a specific pathway of mast cell differentiation/maturation. Instead, it appears that the phenotype of the mast cell populations that develop in the presence of SCF, like that of early hematopoietic cells maintained in SCF (see Section IX,A), may be regulated by the entire mixture of relevant cytokines available to the cell. Elucidating the molecular basis for the distinct patterns of gene expression exhibited by mast cells stimulated simultaneously or sequentially by SCF and other growth factors represents an area of considerable interest, with potentially broad relevance to the larger issues of lineage commitment and differentiation during hematopoiesis.

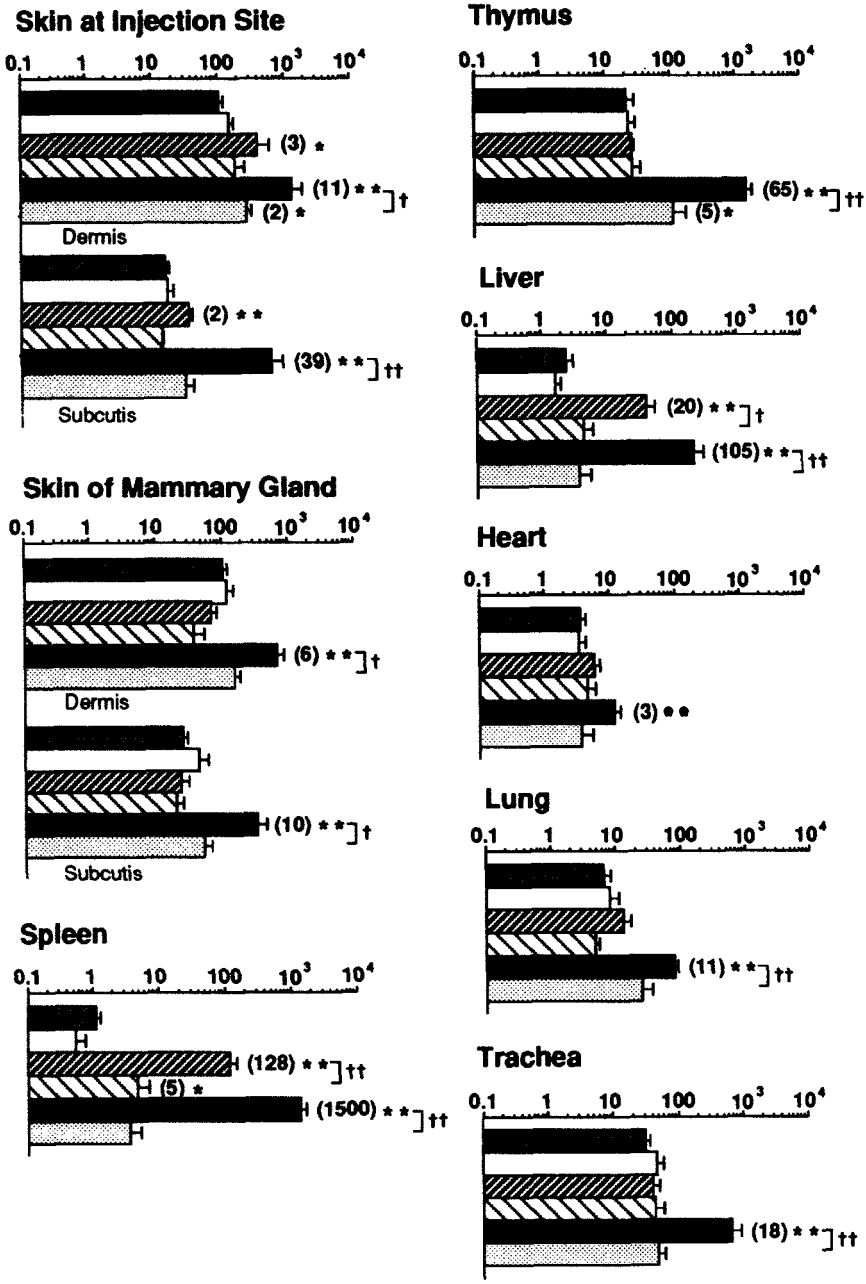
Unlike IL-3, which promotes the development of immature mast cells from mouse bone marrow cells *in vitro* but which is essentially ineffective as a mast cell growth factor in humans, SCF importantly influences mast cell development in nonhuman primates and in humans. Combinations of rhSCF and IL-3 promoted the development of mast cells in cultures of CD34$^+$ human bone marrow cell *in vitro*, but also promoted in parallel the development of many other hematopoietic lineages (Kirschenbaum *et al.*, 1992). As a result, the cultures did not exhibit a net enrichment for mast cells. In contrast, when used as

the only exogenous cytokine, rhSCF induced the development of mast cells from populations of human bone marrow or peripheral blood mononuclear cells (Valent *et al.*, 1992), human umbilical cord blood mononuclear cells (Ishizaka *et al.*, 1992; Mitsui *et al.*, 1993), or human fetal liver cells (Nilsson *et al.*, 1992; Irani *et al.*, 1992). However, the human mast cells that develop in cultures supplemented with soluble rhSCF do not exhibit the full range of phenotypes expressed by human mast cells *in vivo*. For example, these cells express little or no cytoplasmic granule-associated chymase (Ishizaka *et al.*, 1992; Nilson *et al.*, 1992). Whether membrane-associated forms of SCF might differ from soluble SCF in promoting human mast cell development remains to be determined, but the currently available data are certainly consistent with the possibility that additional factors important for the regulation of human mast cell development and phenotype remain to be defined.

Demonstration that a particular cytokine represents a mast cell growth factor *in vitro* does not necessarily indicate that that cytokine will be a mast cell growth factor *in vivo*. However, studies in two species of nonhuman primates indicate that SCF is an important mast cell growth factor *in vivo*. Baboons (*Papio* species) or cynomolgus monkeys (*Macaca fascicularis*) treated subcutaneously with rhSCF for 28 or 21 days, respectively, developed mast cell hyperplasia in many anatomical sites (Galli *et al.*, 1993). However, the magnitude of the effect varied considerably according to anatomical site. In cynomolgus monkeys, treatment with rhSCF at 6.0 mg/kg/day resulted in increases in mast cells in all sites examined except the CNS, with increases ranging from 3-fold, in the heart, to 1500-fold, in the spleen (Fig. 7). But when rhSCF was given at 100 μg/kg/day, a dose that probably will more closely approximate that used clinically in humans, significant elevations of mast cell numbers (by 3- to 20-fold) occurred only in the skin at the injection site, bone marrow, mesenteric lymph node, liver, and spleen.

Remarkably, mast cell numbers in most anatomical sites in monkeys treated with rhSCF for 21 days and then maintained without the growth factor for an additional 15 days were statistically indistinguishable from the baseline numbers observed in vehicle-treated control monkeys (Fig. 7) (Galli *et al.*, 1993). Moreover, the rhSCF-treated monkeys appeared to be clinically well, not only throughout the course of treatment, but also during the period when mast cell populations were declining precipitously to normal levels (Galli *et al.*, 1993).

These findings showed that quantitatively striking systemic expansions and contractions of mast cell populations can occur without clinically obvious adverse effects. The marked and reversible variation in


Skin at Injection Site
0.1 1 10 100 10^3 10^4
(3) *
(11) * *
†
(2) *
Dermis
(2) * *
(39) * *
††
Subcutis
Skin of Mammary Gland
0.1 1 10 100 10^3 10^4
(6) * *
†
Dermis
(10) * *
†
Subcutis
Spleen
0.1 1 10 100 10^3 10^4
(128) * *
††
(5) *
(1500) * *
††
Thymus
0.1 1 10 100 10^3 10^4
(65) * *
††
(5) *
Liver
0.1 1 10 100 10^3 10^4
(20) * *
†
(105) * *
††
Heart
0.1 1 10 100 10^3 10^4
(3) * *
Lung
0.1 1 10 100 10^3 10^4
(11) * *
††
Trachea
0.1 1 10 100 10^3 10^4
(18) * *
††

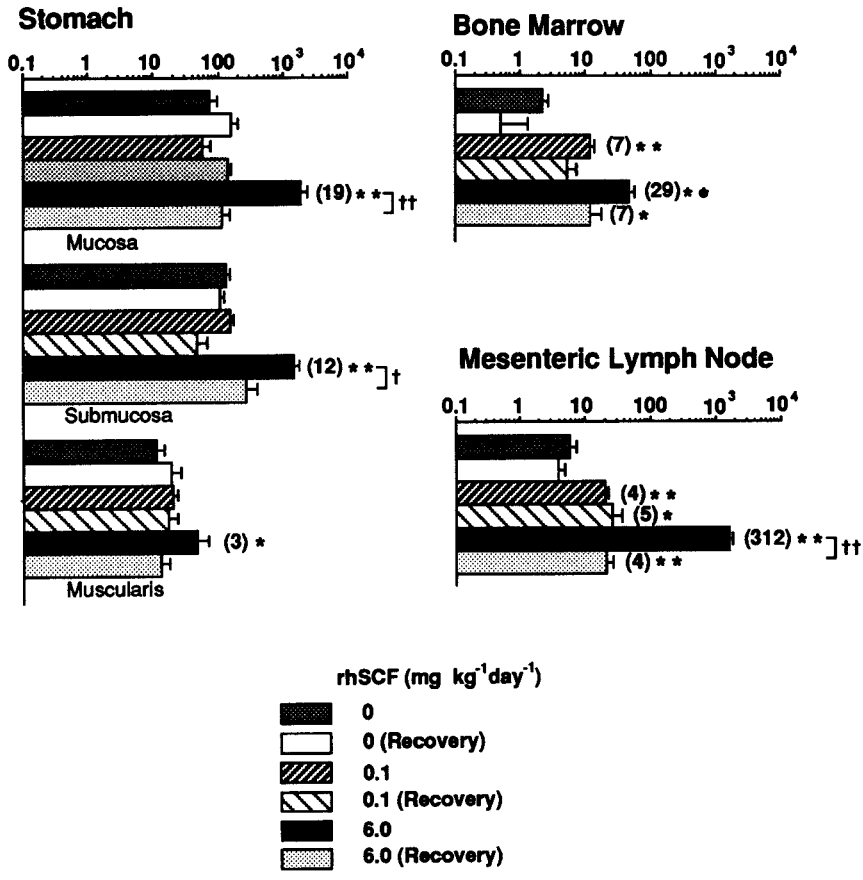


FIG. 7. Mast cell density in various tissues of monkeys treated with rhSCF, which induces reversible mast cell hyperplasia. Numbers of mast cells (cells/mm^2 of tissue, mean ± SEM, shown on a logarithmic scale) are those in various organs/tissues of cynomolgus monkeys (*Macaca fascicularis*) treated for 21 days with twice daily s.c. injections of vehicle or rhSCF (0.1 or 6.0 mg/kg/day). Monkeys were killed for autopsy 1 day (day 22 values) or 15 days (day 36 values, "recovery group") after the last injection of vehicle or rhSCF. * or ** = $p \leq 0.05$ or 0.01 versus corresponding value for vehicle-treated animals; † or †† = $p < 0.05$ or 0.01 for comparisons between day 22 and day 36 values in the same treatment group (Mann–Whitney *U* test, two-tailed). The figures in parentheses refer to the ratio of the mean value in the indicated group to the mean value of the combined day 22 and day 36 vehicle-treated groups. Reproduced from Galli *et al.* (1993) with permission.

mast cell numbers observed in monkeys treated with *exogenous* SCF also raises an interesting question: can changes in the level of expression of *endogenous* SCF explain, at least in part, some of the striking alterations in mast cell numbers that have been noted in association with a variety of reparative responses, immunological reactions, and disease processes, including mastocytosis (reviewed in Galli, 1990, 1993)? We have proposed that all of the currently available evidence suggests that the answer to this question is yes (Galli *et al.*, 1993). Moreover, we have speculated that the great variation in the numbers of mast cells ordinarily present in various normal tissues may largely be determined by the levels of endogenous SCF bioreactivity that are expressed in these different anatomical sites (Galli *et al.*, 1993).

Costa *et al.* (1993) quantified mast cell numbers in skin biopsies obtained from patients enrolled in a phase I study of rhSCF. Treatment of these subjects with rhSCF at 5 to 25 μg/kg/day for 14 days resulted in an ~70% increase in the number of dermal mast cells in skin distant from the SCF injection sites. These represent the first data indicating that rhSCF can induce mast hyperplasia *in vivo* in humans.

B. Mast Cell Function

In addition to providing an excellent model for studying how SCF regulates the development of a single cellular lineage in normal animals, mast cells represent a striking opportunity to analyze the functional consequences of mutations affecting the SCFR or SCF in cells that express the SCFR. Although mice with double-dose mutations that severely affect the production or function of the SCFR ordinarily are virtually devoid of mature mast cells (see Section V,A), large numbers of immature mast cells can readily be generated *in vitro* from the hematopoietic tissues of such *W* mutant mice, e.g., by maintaining the bone marrow or fetal liver cells of these mice in media containing IL-3 (Yung and Moore, 1982; Suda *et al.*, 1985; Nakano *et al.*, 1985). These *in vitro*-derived mast cell populations have been used to isolate and characterize cDNA corresponding to various mutant alleles at *W* (Nocka *et al.*, 1990a; Reith *et al.*, 1990) and to characterize the effects of various *W* mutations on the production and kinase activity of the SCFR (Nocka *et al.*, 1989, 1990a; Reith *et al.*, 1990). These cells also have been used to analyze how mutations involving c-*kit* influence SCF-dependent signal transduction (Rottapel *et al.*, 1991).

As noted earlier (Section VIII,F), the mast cells of *W* mutant mice also were instrumental in investigating a consequence of signaling through the SCFR, which could not have been predicted based on the phenotypic abnormalities expressed by *W* or *Sl* mutant mice: the in-

duction of mast cell secretion and mediator release. The first evidence that rrSCF^{164} can induce mediator release from mouse mast cells was derived from *in vitro* studies (Galli *et al.*, 1991), but the first demonstration that this effect of SCF was SCFR dependent was based on *in vivo* studies that showed that rrSCF^{164} can trigger mast cell activation and a mast cell-dependent inflammatory response when injected intradermally in mice (Wershil *et al.*, 1992b). Mouse mast cell activation by rrSCF^{164} *in vivo* clearly was SCFR dependent, because mast cells expressing the wild-type SCFR were responsive to rrSCF^{164}, whether these cells were present in normal mice or were adoptively transferred into W/W^v mice, whereas cutaneous mast cells of W/W^v origin were completely unresponsive (Wershil *et al.*, 1992b). The unresponsiveness of W/W^v mast cells to rrSCF^{164} did not reflect any general secretory abnormality in these cells, because they are fully competent to undergo activation by IgE and specific antigen (Gordon and Galli, 1990b; Wershil *et al.*, 1992b). SCF also can induce mediator release from mouse peritoneal mast cells (Coleman *et al.*, 1993). The response of rat peritoneal mast cells (Nakajima *et al.*, 1992) or mouse peritoneal mast cells (S. J. Galli, unpublished data) to SCF is enhanced when the challenge is performed in media containing phosphatidylserine.

In addition to being able to induce mouse mast cell mediator release directly, rrSCF^{164} also can significantly augment the magnitude of mouse mast cell mediator release in response to IgE-dependent activation (Coleman *et al.*, 1993). Analyses of isolated human mast cells indicate that concentrations of rhSCF that are similar to the levels of endogenous SCF in the serum of normal subjects (~3 ng/ml) can induce low levels of mediator release from skin (Columbo *et al.*, 1992) but (perhaps) not from lung (Bischoff and Dahinden, 1992) mast cells, and that brief (10 minutes to 1 hour) preincubation of skin (Columbo *et al.*, 1992) or lung (Bischoff and Dahinden, 1992) mast cells with even lower concentrations of rhSCF can significantly enhance the ability of these cells to release mediators in response to FcεRI-dependent stimulation. Notably, however, preincubation of isolated human skin mast cells with rhSCF for ~24 hours *in vitro* did not result in enhancement of IgE-dependent mediator release from these cells (Columbo *et al.*, 1992).

Taken together, the data from mouse (Galli *et al.*, 1991; Wershil *et al.*, 1992b; Coleman *et al.*, 1993) and human (Columbo *et al.*, 1992) mast cells indicate that soluble forms of SCF can directly induce activation of mast cells for secretion of cytoplasmic granule-associated mediators and release of lipid mediators, and that, at even lower con-

centrations, SCF can enhance mediator release in response to activation of the cells through the FcεRI. However, mast cells in tissues *in situ* would be expected to experience a certain baseline level of SCF bioactivity. Indeed, such SCF-dependent activation of the SCFR probably is necessary for the survival of mast cells in normal tissues *in vivo*. What, then, is the biological significance of the effects of SCF on mast cell secretory activity?

One possibility is that SCF/SCFR interactions importantly regulate the extent of mast cell responsiveness, or "releasability," to signals transmitted through the FcεRI. In normal tissues, baseline levels of SCF bioactivity may contribute to setting baseline levels of mast cell secretory responsiveness to activation by IgE and antigen. In contrast, elevations in levels of SCF, such as might occur in association with immunological or pathological processes, could alter the magnitude or nature of the mast cell response to FcεRI-dependent signaling. Although most of the currently available data suggest that processes that increase levels of SCF bioactivity would enhance IgE-dependent mast cell mediator release, this might not always be the case. For example, we have reported that cutaneous sites repeatedly injected with rrSCF^{164} in mice exhibit significantly *less extensive* IgE-dependent mast cell activation than do sites injected with vehicle alone (Ando *et al.*, 1993). Moreover, normal mice injected repeatedly with rrSCF^{164} exhibit significant elevations in mast cell densities, including those in the respiratory system and heart, but do not exhibit significantly enhanced cardiopulmonary responses during IgE-dependent anaphylaxis. Even more remarkably, the rrSCF^{164}-treated mice challenged with the highest dose of specific antigen exhibit fewer deaths due to IgE-dependent anaphylaxis than do the vehicle-treated control mice (1/11 versus 8/11 during the first 60 minutes after antigen challenge, respectively; $p < 0.01$). Although the mechanisms that account for these findings remain to be defined fully, the observations certainly indicate that the effects of SCF on IgE-dependent mast cell secretion may be complex and, depending on the circumstances, may either enhance or suppress mast cell secretory activity and the biological consequences of mast cell activation.

It is of more than passing interest that SCF, a major, if not *the* major, regulator of mast cell development, also can influence the nature of the mast cell response to activation through the FcεRI, the receptor that confers immunological specificity to mast cell activation and that regulates mast cell effector function in IgE-dependent immunological and allergic responses. Although the cross-communication between the SCFR and the FcεRI remains to be characterized in biochemical

terms, there already is evidence that the communication may be bidirectional. Thus, IgE-dependent mast cell activation significantly suppresses the proliferation response of the cell to either SCF or IL-3 (Tsai *et al.*, 1993). Remarkably, activation of mouse mast cells through either the SCFR or the FcεRI induces a similar pattern of changes in mRNA for the early response genes c-*fos* and c-*jun* (Tsai *et al.*, 1993) (see Section VII). Nevertheless, the biological responses of these mast cells to IgE and antigen (i.e., extensive secretion and *suppression* of cytokine-dependent proliferation) or SCF (i.e., enhanced secretion and *induction* of proliferation) are quite different. Accordingly, despite similarities in some aspects of the early cellular responses to signaling via the SCFR or the FcεRI, the full spectra of intracellular signaling that are initiated at these two receptors must be distinct. Elucidating further similarities or differences between SCFR-dependent and FcεRI-dependent signaling in mast cells may be of interest not only because this work will enhance our understanding of mast cell biology, but because such work may have relevance to the role of SCF/SCFR interactions in influencing the function of other cellular lineages that express the SCFR.

In addition to influencing the secretory activity of mast cells stimulated through the FcεRI, studies in mouse mast cells *in vivo* (Wershil *et al.*, 1992b) or *in vitro* (Coleman *et al.*, 1993) or in human skin mast cells *in vitro* (Columbo *et al.*, 1992) indicate that soluble SCF can directly promote mast cell secretion and mediate release. What is the biological significance of these observations? Normal tissues do not ordinarily exhibit significant mast cell degranulation or express inflammatory responses. These findings indicate that physiological interactions between SCFR expressed by mast cells and endogenous forms of membrane-associated or soluble SCF do not generally result in extensive mast cell degranulation and secretion.

On the other hand, low levels of mast cell activation by SCF may have a role in both the development and the function of this cell type. Activation of mouse mast cells via the FcεRI or by other mechanisms induces the cells to develop increased levels of mRNA for several cytokines and/or to secrete the products (Plaut *et al.*, 1989; Wodnar-Filipowicz *et al.*, 1989; Burd *et al.*, 1989; Gordon and Galli, 1990a; Galli, 1993). Some of these cytokines, such as IL-3 and IL-4, can augment mouse mast cell proliferation whereas others, such as GM-CSF and IFN-γ, can suppress mast cell proliferation (reviewed in Galli, 1990; Galli *et al.*, 1992). Keegan *et al.* (1991) reported that stimulation of endogenous or transfected growth factor receptors with their cognate ligands could enhance IgE-dependent cytokine produc-

tion by mouse mast cells. Razin *et al.* (1991) reported evidence that mouse mast cells can develop increased levels of mRNA for IL-3 after establishing contact with 3T3 cells *in vitro*. As noted earlier, several lines of evidence indicate that the ability of 3T3 fibroblasts to induce mouse mast cell proliferation and maturation required an interaction between SCF associated with the fibroblasts and the SCFR expressed by the mast cells. Accordingly, one of the mechanisms by which SCF influences mast cell development may be to induce mast cells to produce other cytokines with autocrine effects on mast cell proliferation or maturation.

Moreover, there has been a long history of speculation that one physiological role of the mast cell is to release constitutively very low levels of mediators that, when released rapidly in larger quantities, result in the development of an inflammatory response (reviewed in Galli *et al.*, 1984). Findings demonstrating that SCF can induce mast cell mediator release do not prove that physiological interactions between SCF and its receptor induce low levels of mast cell mediator release in normal tissues, but they are compatible with this hypothesis.

XI. Melanocyte Biology

Defects in skin pigmentation represent a major class of phenotypic abnormalities in *W* or *Sl* mutant mice (Silvers, 1979a). Nocka *et al.* (1989) found high levels of expression of SCFR mRNA, as well as SCFR protein, in PMA-dependent melanocyte cell lines derived from normal newborn mice. Orr-Urtreger *et al.* (1990) detected high levels of expression of SCFR mRNA in individual cells in the subepidermal space and developing hair follicles of midgestation and older mouse embryos; at least some of these cells were considered to represent melanocyte precursors.

Matsui *et al.* (1990) showed that SCF mRNA was expressed in the dorsal region of day 10.5 mouse embryo somites, and at later intervals in the dermis and hair follicles. In a subsequent *in situ* hybridization study, Manova and Bachvarova (1991) found that SCFR mRNA expression was first detectable in presumptive melanoblasts in the cervical region of day 10 embryos. By day 13.5 p.c., SCFR mRNA-positive presumptive melanoblasts appeared to have greatly increased in number and were found in the skin from the dorsal to ventral midline, and some of these cells were located in the epidermis. At birth, many labeled cells were present in the dermis, epidermis, and hair follicles. According to staining of parallel specimens of skin with toluidine blue, some of the SCFR mRNA-positive cells in the dermis may have repre-

sented mast cells, but mast cells do not ordinarily occur in the epidermis or within hair follicles.

Taken together, this work indicated that interactions between the SCFR and its ligand may contribute to the migration of melanoblasts, and also to their proliferation. However, Manova and Bachvarova (1991) noted that, in 1949, Russell had reported that melanocytes in $W^v/+$ mice contained less pigment per cell than did melanocytes in +/+ mice, and therefore suggested that the SCFR might also influence differentiation in this lineage.

To determine precisely when the SCFR is required for important steps in melanocyte development, Nishikawa *et al.* (1991) generated a rat monoclonal antibody (ACK-2) that can block mouse SCFR function and administered the antibody to mice in an attempt to interfere with melanocyte development or function either during embryogenesis or postnatally. This approach was especially attractive because administration of ACK-2 at appropriate intervals resulted in patterns of coat color dilution in the treated mice, thus providing clear evidence of the biological consequences of ACK-2 treatment on melanocytes. In contrast, there are no satisfactory markers for unequivocally identifying melanoblasts by light microscopy during their migration from the neural crest to the hair follicle.

Nishikawa *et al.* (1991) found that the effect of ACK-2 administration on melanocyte development was critically dependent on the time of its administration to the pregnant mouse: injection later than day 15.5 p.c. had no detectable effect and injection earlier than day 10.5 or 11.5 p.c. had little or no effect. In contrast, injection on day 13.5 p.c. resulted in virtually complete depigmentation of the offspring and injection on day 14.5 p.c. caused striking but very variable patterns of partial depigmentation. Immunohistochemical studies with ACK-2 showed that numbers of mesodermal $SCFR^+$ cells greatly increased on days 14.5 to 15.5 p.c., with $SCFR^+$ cells first appearing in the dorsolateral epidermis on day 15.5 p.c. Administration of ACK-2 on day 14.5 resulted in a markedly diminished number of $SCFR^+$ cells in the skin, as well as in depigmentation. In contrast, antibody administered on day 15.5 p.c. bound to $SCFR^+$ cells in the epidermis, but had no effect on coat pigmentation. Accordingly, Nishikawa *et al.* (1991) concluded that the proliferation of melanocyte precursors in the mesodermal layer, and their subsequent migration into the epidermis, are critically dependent on the SCFR, whereas melanocytes within the epidermis are much less SCFR dependent.

Nishikawa *et al.* (1991) also showed that injection of ACK-2 into an adult C57BL/6 mouse 1 day after shaving an area of the animal's skin

resulted in depigmentation of the regenerated hair. Nishikawa *et al.* (1991) concluded that the SCFR is also required for the melanocyte proliferation that is activated during the initial phase of the hair cycle.

There have been relatively few studies of the effects of SCF on melanocytes *in vitro*. SCF transiently prolongs the *in vitro* survival of melanocytes derived from human foreskin, but cannot, by itself, induce proliferation of these cells (Funasaka *et al.*, 1992). However, SCF can induce human melanocyte proliferation when administered together with PMA. Melanocytes exposed to SCF exhibit autophosphorylation of the SCFR, which is followed by tyrosine phosphorylation of a set of intracellular substrates that appear to be different than those utilized in mast cells. The possible role of SCF/SCFR interactions in melanoma is discussed in Section XIV,D.

XII. Germ Cell Development

In normal mice, primordial germ cells (PGCs) are first identifiable at ~7 days p.c., when they appear as large alkaline phosphatase-positive cells in the extraembryonic mesoderm just proximal to the primitive streak (Ginsburg *et al.*, 1990). At ~8 days p.c., 10 to 100 PGCs are present at the base of the allantois next to the caudal primitive streak (Heath, 1978). Over the next 4 days, PGC number increases rapidly to 2500–5000 and the cells migrate from the hindgut to the gonadal ridge. Proliferation of PGCs occurs during their migration and for 1–2 days after colonization of the gonadal ridge. By 12.5 days p.c., the gonadal ridge has undergone sexual differentiation into the male or female gonad (Eddy *et al.*, 1981).

In mice homozygous for severe (lethal) *W* alleles, germ cells can be identified at day 8 p.c. but do not subsequently increase in number (Mintz and Russell, 1957). Sl/Sl^d mice of both sexes also essentially lack germ cells in the gonads (Bennett, 1956; McCoshen and McCallion, 1975; Green, 1981). Bennett (1956) attributed this phenotype to impaired migration of primordial germ cells from the epiblast to the genital ridges, but McCoshen and McCallion (1975) could not confirm this mechanism and instead attributed the reduced number of PGCs in the mutant embryos to diminished proliferation and/or excessive cell death. Several lines of evidence, including analyses of the effects of SCF on PGC *in vitro* (Dolci *et al.*, 1991; Godin *et al.*, 1991; Matsui *et al.*, 1991) and of the effects of the Sl^{17H} allele on PGC development *in vivo* (Brannan *et al.*, 1992), indicate that normal SCF/SCFR function is required for the survival and/or proliferation of PGCs.

However, the effects of *W* or *Sl* mutations on the reproductive sys-

tem are quite complex and indicate that the SCFR and its ligand influence germ cell biology in the postnatal gonads, as well as in embryonic development. Thus, juvenile W/W^v or W^v/W^v mice not only have profound reductions in the number of germ cells, but also exhibit diminished rates of development of oocytes and spermatogenic cells (Coulombre and Russell, 1954). Moreover, some *W* mutations have distinct effects on germ cell development in males or females, indicating that SCR receptor-dependent functions vary according to sex in phases of germ cell development after the primordial germ cell stage (Geissler *et al.*, 1981). Nishimune *et al.* (1980, 1984) confirmed that *Sl* mutations reduce numbers of testicular germ cells but also showed that these mutations interfere with meiotic division and with the differentiation of type A spermatogonia to type B or intermediate spermatogonia. And one *Sl* allele, Sl^t, appears to cause infertility in females because of an intrinsic defect in ovarian stromal cells that results in growth arrest of ovarian follicles (Kuroda *et al.*, 1988). Notably, the ovaries of *WB-*Sl/Sl^t and *WB-*+/+ mice have similar numbers of primordial follicles, consisting of oocytes surrounded by a single layer of granulosa cells (Kuroda *et al.*, 1988). This finding indicates that Sl/Sl^t mice may express less impairment of SCF function than do Sl/Sl^d mice. Indeed, other phenotypic abnormalities of Sl/Sl^t mice are also less striking than those in the Sl/Sl^d animals. For example, male Sl/Sl^t mice exhibit normal fertility (Kohrogi *et al.*, 1983). Sl/Sl^t mice exhibit nearly complete depigmentation of the coat, but have partial pigmentation of the ear pinnae, and have a moderate anemia and mast cell deficiency (Kohrogi *et al.*, 1983).

In contrast, Sl^{17H}/Sl^{17H} females exhibit a significant reduction in primordial and growing oocytes, probably due to effects of Sl^{17H} on PGC development, but are fertile (Brannan *et al.*, 1992). Sl^{17H}/Sl^{17h} males also exhibit greatly reduced PGCs, but in addition exhibit virtual cessation of sperm development after the first wave of spermatogenesis (Brannan *et al.*, 1992). Accordingly, the splicing defect and abnormal cytoplasmic tail of SCF in Sl^{17H}/Sl^{17H} mice appear to impair both embryonic germ cell development and male postnatal germ cell development.

The localization and expression of the SCF receptor and its ligand during germ cell development have been analyzed in detail. *In situ* hybridization studies demonstrated c-*kit* mRNA in primordial germ cells in the genital ridges of midgestation mouse (day 12.5 p.c.) embryos (Orr-Urtreger *et al.*, 1990). In a subsequent study, Manova and Bachvarova (1991) associated c-*kit* mRNA expression with PGCs located at the base of the allantois at day 7.5 p.c., at close to the earliest

time PGCs can be identified. Expression in germ cells continued during early stages of proliferation and migration to the gonad. Notably, c-*kit* mRNA expression was also detected in the tissues adjacent to migrating germ cells. Expression in gonia in ovaries and testes increased in association with their proliferation then decreased as the female germ cells entered meiosis and the male germ cells became quiescent.

In adult gonads, Orr-Urtreger *et al.* (1990) detected c-*kit* mRNA in the ovaries in primary and antral follicles of 2-week-old mice and in the mature Graafian follicles of a 2-month-old mouse; highest levels of expression were in the oocytes. In subsequent studies, Manova *et al.* (1990) confirmed the localization of c-*kit* transcripts to oocytes in juvenile mice, with a pattern of expression that appeared to correlate strongly with oocyte growth. Little c-*kit* mRNA expression was detected in two-cell embryos and none in blastocytes. Manova *et al.* (1990) also detected significant c-*kit* mRNA expression in ovarian somatic cells, a pattern first detected 14 days after birth and at increased intensity on day 17. Expression was thought to be associated with lipid-containing cells of the theca interna. A moderate level of labeling also was detected in the corpora lutea of cycling adults or at 10 days of pregnancy.

Immunohistochemical analysis with a rabbit anti-SCFR antiserum detected SCFR protein on primordial, growing, and mature oocytes and on one- or two-cell embryos, but little was detected on blastocytes (Manova *et al.*, 1990). Horie *et al.* (1991), using the ACK-2 anti-SCFR monoclonal antibody, confirmed the expression of SCFR protein on the surface of oocytes, beginning at birth. However, no SCFR protein was detected in PGCs undergoing mitosis in fetal ovaries on day 15.5 p.c. Weak expression of SCFR protein was detected on single-cell embryos but expression diminished with cell division and was absent on four-cell embryos. Horie *et al.* (1991) detected a high level of SCFR expression on the surface of oocytes arrested in the diplotene stage of meiotic prophase, and found that expression declined with ovulation and the resumption of meiotic maturation. These findings suggested that the SCFR/SCF system might contribute to meiotic arrest, as well as to oocyte growth and maturation. However, administration of the ACK-2 neutralizing antibody to the SCFR to mice *in vivo* had no detectable effect on occyte maturation or ovulation (Yoshinaga *et al.*, 1991).

In the testes, Orr-Urtreger *et al.* (1990) detected c-*kit* mRNA in interstitial Leydig cells but not in spermatogonia or Sertoli cells. In contrast to Orr-Urtreger *et al.* (1990), Manova *et al.* (1990) detected

c-*kit* mRNA expression in spermatogonia, beginning at 6 days after birth. Expression during the cycle of the seminiferous epithelium extended at least from A_2 spermatogonia through type B. Using immunohistochemistry, Yoshinaga *et al.* (1991) confirmed SCFR expression on differentiating type A, intermediate, and type B spermatogonia, and on the earliest preleptotene spermatocytes, but not on later spermatocytes or spermatids, suggesting that the SCFR is down-regulated on entering the meiotic cycle. Yoshinaga *et al.* (1991) also detected high levels of SCFR expression by interstitial Leydig cells but not by Sertoli cells. However, Sertoli cells represent a potential source of biologically active SCF (Tajima *et al.*, 1991). Yoghinaga *et al.* (1991) showed that treatment of mice with the ACK-2 neutralizing antibody to the SCFR completely blocked mitosis of mature (differentiating) type A spermatogonia and resulted in the depletion of these cells from the testes, but did not affect the mitosis of gonocytes and primitive type A spermatogonia, or the meiosis of spermatocytes. These results strongly support the hypothesis that the SCFR is necessary for the survival and/or differentiation of type A spermatogonia.

XIII. The Nervous System

Detection of mRNA for the SCFR and SCF in the central nervous system represented among the earliest and most surprising findings obtained after the cDNAs for these genes were cloned (Yarden *et al.*, 1987; Geissler *et al.*, 1988b; Majumder *et al.*, 1988; Qiu *et al.*, 1988; Matsui *et al.*, 1990). These findings were unexpected because defects in CNS structure or function had not been reported among the constellation of phenotypic abnormalities expressed by *W* or *Sl* mutant mice.

The initial descriptions of the distribution of SCFR mRNA in the central nervous system were based on Northern analyses (Yarden *et al.*, 1987; Geissler *et al.*, 1988b; Majumder *et al.*, 1988), whereas the first identification of SCF mRNA in the central nervous system was an *in situ* hybridization analysis of the mouse embryo (Matsui *et al.*, 1990). Subsequently, several groups used *in situ* hybridization to perform detailed analysis of the distribution of SCR and/or SCFR mRNA in embryonic mice (Orr-Urtreger *et al.*, 1990; Motro *et al.*, 1991; Keshet *et al.*, 1991), adult mice (Orr-Urtreger *et al.*, 1990; Morii *et al.*, 1992), or rats (Hirota *et al.*, 1992). Although a detailed description of the results of these studies is beyond the scope of this chapter, a few points deserve emphasis.

In both embryos and adult mice, SCFR mRNA was detected in the cerebellum, hippocampus, and telencephalon (Orr-Urtreger *et al.*,

1990), whereas SCF mRNA appeared in the brain and spinal cord, with levels increasing after birth (Matsui *et al.*, 1990). A detailed examination of the expression of SCFR and SCF mRNA in the cerebellum of adult mice detected SCFR mRNA in the molecular layer of the cerebellum and SCF mRNA at the boundary of the molecular and granular layers. Several lines of evidence, including the lack of expression of SCF mRNA in the cerebellum of lurcher (*Lc*/+) mice that lack Purkinje cells, indicated that the cells that expressed SCF were Purkinje cells, and that the SCFR mRNA-positive cells were neurons representing basket cells and stellate cells. Because such neurons form suppressive neurojunctions with Purkinje cells, Morii *et al.* (1992) proposed that SCF/SCFR interactions may have a role in the formation of such junctions.

An *in situ* hybridization study of the expression of SCF and SCFR mRNA in the CNS of adult rats provided further evidence that SCF/SCFR interactions may have a role in the formation of certain synaptic connections (Hirota *et al.*, 1992). Thus, in addition to the expression of SCF mRNA by Purkinje cells and SCFR mRNA by cerebellar basket/stellate cells, Hirota *et al.* (1992) identified other examples in which pairs of neurons that have been reported to form synaptic connections express either SCF or SCFR mRNA. In the olfactory bulb, tufted cells expressed the SCFR mRNA, whereas mitral cells and periglomerular cells expressed SCF mRNA; the principal sensory and the spinal nuclei of the trigeminal nerve expressed SCFR mRNA, whereas the ventroposterior nucleus of the thalamus expressed SCF mRNA; certain cells in the cerebral cortex and the caudate putamen expressed mRNA for the SCFR, whereas the paracentral and centrolateral nuclei of the thalamus expressed mRNA for SCF (Hirota *et al.*, 1992).

However, if SCF/SCFR interactions do contribute to CNS development or function, why have abnormalities in CNS structure or function not been identified in *W* or *Sl* mutant mice? As noted by Hirota *et al.* (1992), *W* or *Sl* mutants that totally lack the extracellular domain of either the SCFR or SCF do not survive into adulthood. This complicates analysis of their CNS development or function. On the other hand, if these mice can be rescued (e.g., by transplanting normal bone marrow cells into *W*/*W* mice), perhaps they would manifest neurological problems. Notably, *W* or *Sl* mutant mice that have at least one allele encoding a form of SCF or the SCFR that retains the extracellular domain (such as W/W^v or Sl/Sl^d mice) do survive into adulthood. This finding is consistent with the hypothesis that the SCF/SCFR interactions necessary for normal CNS development or function do not depend on normal signaling through the tyrosine kinase domain of the

SCFR, but may require only adherence, or chemotaxis/haptotaxis mediated by functionally adequate SCFR.

Alternatively, SCF and the SCFR may form part of a set of molecules involved in CNS development or function that express considerable redundancy in action: these other molecules may be able largely to compensate for a lack of SCF or SCFR in *W* or *Sl* mutant mice. Another possibility is that proper CNS development can occur because of the expression in this site of alternative ligands for the SCFR, or alternative receptors that can interact with SCF.

Finally, it is possible that *W* or *Sl* mutant animals do express CNS structure or function abnormalities that have not yet been recognized. In this context, it is intriguing that piebaldism and mental retardation have been reported in a child with a deletion of 4q11–4q21 as its only chromosomal abnormality (Lacassie *et al.*, 1977), and that deafness, impaired motor development, and mental retardation have been reported in a child presumed to be homozygous for the piebald trait, but in whom c-*kit* was not analyzed (Hulten *et al.*, 1987). However, it is not known whether these patients' abnormalities were related to defects associated with the SCFR, or to other problems. Perhaps one of the difficulties in detecting CNS abnormalities in *W* or *Sl* mutant animals is that such SCFR/SCF-related problems are subtle. In this regard, it is of interest that other growth factors that are ligands for RTKs, such as epidermal growth factor (Abe *et al.*, 1991; Abe and Saito, 1992; Ishiyama *et al.*, 1991) or fibroblast growth factor (Ishiyama *et al.*, 1991), can enhance induction of long-term potentiation of receptor-mediated responses in certain regions of the brain, such as the hippocampus or dentate gyrus.

XIV. Neoplasia

A. Introduction

There is growing evidence that aberrant expression of the SCFR signaling pathway may play a role in the etiology of neoplasms in mice and humans. The oncogenic potential of the v-*kit* product, which represents a constitutively active form of SCFR truncated at both amino and carboxy ends, provided the first evidence that constitutive activation of the SCFR signal transduction pathways was potentially oncogenic (Besmer *et al.*, 1986; Majumder *et al.*, 1990). Prior to the cloning of SCF, Lev *et al.* (1990) demonstrated that NIH3T3 cells transfected with a chimeric cDNA encoding a fusion protein comprising the extracellular domain of the EGF receptor and the transmembrane and intracellular domains of the SCFR exhibited a proliferative response to

EGF and acquired the ability to grow in soft agar in the presence of EGF. This finding implied that the ligand for the c-kit receptor (SCFR) might also transmit a strong mitogenic signal that could elicit some characteristics of oncogenic transformation. In accord with this hypothesis, Alexander *et al.* (1991) showed that NIH3T3 fibroblasts that had been infected with a c-*kit* retroviral expression construct exhibited a transformed phenotype *in vitro*. Although the transformed phenotype was most apparent in the presence of exogenous SCF, a low level of colony formation in soft agar was observed even in the absence of added SCF.

Given the previously established oncogenic potential of other members of the RTK type III family (Ullrich and Schlessinger, 1990; Cantley *et al.*, 1991), these data indicated that aberrant SCFR signal transduction could play a role in the etiology of neoplasia. The possible mechanisms could include (1) elevated expression of the wild-type SCFR, leading to ligand-independent signal transduction; (2) the production of altered forms of the SCFR (e.g., that encoded by v-*kit*) that express ligand-independent kinase activity; (3) autocrine, juxtacrine, or paracrine SCF-mediated SCFR signal transduction arising from either the ectopic expression of c-*kit* in tissues that normally express *SCF* or the ectopic expression of SCF in tissues which normally express the SCFR; or (4) in some cell types in which SCF/SCFR interactions may limit the growth of the $SCFR^+$ cells, changes that reduce signaling through the $SCFR^+$ could lead to increased growth of $SCFR^+$ cells.

B. Hematopoietic Tumors

W and *Sl* mutations can have profound effects upon lymphopoiesis in the aging mouse, suggesting an important potential role for the SCFR signaling pathway in leukemogenesis. The incidence of lymphocytic leukemia is about 50-fold higher and appears earlier in $B6C3F_1$-W^x/W^v (W^x/W^v) mice than in age-matched normal control mice (roughly 5% of W^x/W^v versus 0.1% of +/+ mice) (Murphy, 1977; Russell, 1979). The incidence of lymphocytic leukemias is also elevated in Sl/Sl^d mice, with 37% of these mutant mice exhibiting disease at an average age of 1 year, in contrast to only 5% of their normal littermates exhibiting disease at an average age of about 3 years (Murphy, 1977; Russell, 1979). These findings, which apparently have not been further pursued, take on special significance in light of the discovery of the allelism between *W* and c-*kit* and between *Sl* and *SCF* and the role for the SCFR signal transduction pathway in the proliferation of normal and leukemic hematopoietic cells (Section IX and below).

SCFR mRNA and protein are expressed in the blast cells of patients with acute myeloblastic leukemia (AML) but not in blasts from patients with acute lymphoblastic leukemia (ALL) or in myeloid blasts derived from normal marrow (Wang *et al.*, 1989; Lerner *et al.*, 1991). More recent studies have confirmed that c-*kit* expressed by leukemic blasts in the majority of AML cases (Ikeda *et al.*, 1991; Broudy *et al.*, 1992; Carlesso *et al.*, 1992; Goselink *et al.*, 1992). SCF alone can induce a proliferative response in some AML cells *in vitro* (Ikeda *et al.*, 1991; Wang *et al.*, 1991; Broudy *et al.*, 1992; Carlesso *et al.*, 1992) and SCF can synergize with GM-CSF, G-CSF, and/or IL-3 in stimulating the *in vitro* colony formation or proliferation of AML cells (Ikeda *et al.*, 1991; Broudy *et al.*, 1992; Carlesso *et al.*, 1992; Goselink *et al.*, 1992). SCF also can synergize with IL-3 and/or GM-CSF in promoting colony formation from the CML cells of patients in the chronic phase of the disease (Goselink *et al.*, 1992).

In the leukemic blasts and cell lines derived from some AML patients, the SCFR was shown to be constitutively active (i.e., autophosphorylated) and capable of further activation by stimulation with exogenous SCF (Kuriu *et al.*, 1991). Furthermore, the proliferation of the MO7E human myeloid leukemia cell line appears to be dependent on activation of the SCFR (Kuriu *et al.*, 1991). Ratajczak *et al.* (1992a) reported that attempts to block the growth of CFU-GM from several patients with AML or ALL using a c-*kit* antisense oligonucleotide strategy were relatively ineffective, even though the same approach successfully inhibited the growth of CFU-GM from patients with CML or polycythemia vera. Pietsch *et al.* (1991) reported that some c-*kit*-positive human AML cell lines coexpress *SCF*, suggesting that an SCF/SCFR autocrine growth loop could be involved in their proliferation.

Putative rearrangements of c-*kit* were identified in the AML blasts of 4/30 patients examined by Southern analysis (Cheng *et al.*, 1991). However, the consequences for the expression of the rearranged gene have not yet been reported. Therefore, although intriguing, the relevance of these mutations to the transformed phenotype has yet to be confirmed.

In summary, these results indicate that signal transduction through the SCFR may play a role in the etiology of some but not all cases of AML or CML in humans. However, in those cases where this pathway appears to be involved, further work will be required to determine the molecular basis for the activation of SCFR signal transduction and to determine whether or not these findings have prognostic or therapeutic significance. Notably, the number of SCFRs on AML blasts may actually be the same or lower than that on normal blasts and the

numbers of SCFRs expressed by AML cells did not appear to correlate with the *in vitro* proliferative response of these cells to SCF (Broudy *et al.*, 1992). Moreover, SCF plus IL-3 and/or GM-CSF induced erythroid colony formation from bone marrow cells of patients with CML or myelodysplastic syndromes, and SCF plus IL-3 and/or GM-CSF, or SCF and EPO, appeared to induce preferentially the short-term *in vitro* development of normal as opposed to leukemic hematopoietic progenitor cells from the bone marrow of patients with myelodysplastic syndromes (Goselink *et al.*, 1992). Also, when compared to IL-3 or GM-CSF, SCF is a relatively weak stimulus for the *in vitro* clonogenic growth of lineage-restricted leukemic precursor cells (Goselink *et al.*, 1992). Taken together, these findings suggest that SCF may be clinically useful to stimulate normal hematopoiesis in patients with AML or myelodysplastic syndromes (Goselink *et al.*, 1992).

C. Mast Cell Leukemia/Mastocytosis

Rottapel *et al.* (1991) reported that the factor-independent tumorigenic murine mastocytoma cell line P815 expresses a constitutively active SCFR. They demonstrated that P815 cells do not express *SCF*, but did not provide further insight concerning the molecular basis for the constitutive SCFR activity in this cell line. Furitsu *et al.* (1992) discovered that the factor-independent HMC-1 human mast cell leukemia cell line also expresses constitutive SCFR kinase activity in the absence of any endogenous or exogenous SCF protein. Moreover, HMC-1 c-*kit* exhibited two point mutations in the region encoding the cytoplasmic domain of the SCFR. These findings indicate that a variant c-*kit* allele in HMC-1 encodes a constitutively active form of the SCFR, although the inferred role of this constitutive kinase activity in the factor-independent growth or leukemogenicity of the HMC-1 cell line was not reported. It has been proposed that abnormalities of SCF or the SCFR might contribute to some cases of mastocytoma or systemic mastocytosis (Galli, 1993). Longley *et al.* (1993) reported immunohistochemical evidence for an altered distribution of SCF in the skin of three patients with cutaneous mastocytosis, suggesting that some forms of mastocytosis may represent reactive hyperplasias of mast cells rather than neoplasms.

D. Melanoma

The deficiency of cutaneous melanocytes in *W* mutant mice might be expected to provide these animals with at least one selective advantage—i.e., a reduced risk of melanoma. However, Larue *et al.* (1992) recently reported the spontaneous malignant transformation of

melanocytes explanted from the skin of young W^f/W^f mice. Of 10 cultures containing W^f/W^f melanocytes (versus none of more than 50 cultures from normal controls), 3 cultures gave rise to transformed melanocytes shortly after the first few passages. These cells grew in low serum concentrations, formed foci *in vitro*, and gave rise to invasive melanomas when injected into nude mice. The W^f mutation comprises a point mutation in the c-*kit* kinase domain that causes an amino acid substitution at position 816 in the central core of the kinase domain of the SCFR, but which only mildly impairs the kinase activity of the SCFR (Larue *et al.*, 1992; Tsujimura *et al.*, 1993). The mechanism responsible for the spontaneous *in vitro* transformation of W^f/W^f melanocytes is not yet understood.

To our knowledge, spontaneous melanomas have not yet been described in intact W^f/W^f or other *W* mutant mice. However, it is possible that these mutants may be susceptible to the induction of melanomas or other tumors *in vivo* using radiation or chemical tumor promoters. Regardless, the *in vitro* findings suggest that c-*kit* mutations may represent a step in one multistep pathway to melanoma.

Nocka *et al.* (1989) originally reported that c-*kit* mRNA was expressed in normal murine melanocytes but not in two cell lines established from spontaneous murine melanomas. Lassam and Bickford (1992) extended this work and found that cell lines derived from spontaneous or UV light-induced murine melanomas, or melanocytes transformed by transfection with dominant oncogenes, expressed little or no c-*kit* mRNA. They also reported that SCFR mRNA and protein could be detected in cultured normal melanocytes isolated from human foreskin specimens or from skin biopsies from adult compound or congenital nevi, but not in the majority of malignant human melanoma cell lines they examined. Funasaka *et al.* (1992) reported that five of eight human melanoma cell lines expressed c-*kit* mRNA, but that the SCFR was not constitutively activated in these cells. Nor did SCF alone induce proliferation, a cascade of protein phosphorylation, or MAP kinase activation in the majority of cells cultured from primary modular or metastatic melanoma (Funasaka *et al.*, 1992). Taken together, these studies indicate that the majority of transformed mouse or human melanocyte cell lines examined do not require SCFR signal transduction for their survival or proliferation.

It is not yet clear whether the reduced c-*kit* expression in melanoma cells is causally related to the transformation event or is a consequence of dysregulated melanocyte gene expression. Nevertheless, these findings indicate that constitutive SCFR activation is probably not involved in the etiology of the majority of human or mouse melanomas.

However, human melanomas may comprise a phenotypically heterogeneous group of tumors with respect to the expression of c-*kit*. In fact, Turner *et al.* (1992) recently reported a human melanoma cell line (HTT144) that expresses both c-*kit* and *SCF* genes, displays both proteins on the cell surface, and secretes SCF into the culture medium, suggesting that the growth of this and perhaps other melanoma cell lines may involve autocrine stimulation through the SCFR.

E. Gonadal Tumors

As they age, the ovaries of mice carrying two dominant *W* mutations (e.g., W^v/W^v, W^x/W^v, and W^j/W^v) become progressively more depleted of their already genetically reduced population of developing follicles (Russell and Fekete, 1958). Subsequently, the germinal epithelium of the ovaries forms tubular adenomas and eventually gives rise to granulosa cell tumors and luteomas (Murphy and Russell, 1963). The etiology of these tumors is presumed to involve the effects of excessive levels of pituitary gonadotrophins, which are produced in response to the reduced levels of follicular estrogens. However, this hypothesis has yet to be definitely proved. In the context of recent evidence that a large proportion of human germ cell tumors express c-*kit* (see later), it is reasonable to propose that germ-line $W^{c\text{-}kit}$ mutations may play a more direct role in predisposing gonadal tissues for neoplastic transformation.

High levels of c-*kit* transcripts have been detected in a large percentage of human seminomas, but only infrequently in nonseminoma germ cell tumors examined in the same studies (Strohmeyer *et al.*, 1991; Murty *et al.*, 1992; Matsuda *et al.*, 1993). Expression of c-*kit* has not yet been detected in the limited examples of ovarian cancers or in other cancers of the genitourinary system or uterus (Matsuda *et al.*, 1993).

No evidence of amplification or other gross rearrangements in the c-*kit* gene was detected in any of the gonadal tumors so far examined. However, a recent cytogenetic and molecular genetic analysis for loss of constitutional heterozygosity at loci on chromosome 12 in human male germ cell tumors identified sites of candidate tumor suppressor genes on the long arm of chromosome 12 (12q13 and 12q22). One of these regions includes the *SCF* locus (Murty *et al.*, 1992). This study also confirmed the expression of c-*kit* in a large percentage of seminomas (30%) and also noted the expression of *SCF* in 3% of seminomas and 50% of nonseminomas. However, Murty *et al.* (1992) found no evidence for the coexpression of *SCF* and c-*kit* in a single tumor.

The relationship, if any, between the dysregulated expression of *SCF* in some germ cell tumors and the candidate tumor suppression

gene that maps in the *SCF* region of chromosome 12 has yet to be established.

F. Brain Tumors

In their initial analysis of the distribution of c-*kit* expression in human tissues, Yarden *et al.* (1987) noted particularly high levels of expression in the A172 human glioblastoma cell line. Matsuda *et al.* (1993) reported weak expression of SCFR by immunohistochemical staining in each of two human glioblastomas but not in the single examples of astrocytoma or medullablastoma examined. Because c-*kit* is also expressed by normal glial cells in fetal and adult human brains (see Section XIII), elucidation of the role, if any, of c-*kit* expression in the etiology of glioblastoma awaits further analysis. Two preliminary reports have analyzed the expression of c-*kit* or *SCF* in neuroblastoma. One study of 8 human neuroblastoma cell lines found that the cells rarely expressed in SCFR on their surface and did not proliferate in response to SCF (Beck *et al.*, 1993). The other study detected coexpression of c-*kit* and *SCF* by RT-PCR in 16/16 human neuroblastoma cell lines and in 8/18 human neuroblastoma tumors (Cohen *et al.*, 1993).

G. Lung Cancer

Several histological types of lung cancer appear to express c-*kit* mRNA and SCFR protein (Sekido *et al.*, 1991; Matsuda *et al.*, 1993). c-*kit* mRNA is expressed abundantly in small cell lung carcinoma (SCLC) (Sekido *et al.*, 1991; Hibi *et al.*, 1991; Matsuda *et al.*, 1993). SCFR protein was detected on 56% (14/25) of SCLC tumors, on 75% (3/4) of large cell lung carcinomas, on 30% (3/10) of squamous cell carcinomas, and on 1/11 adenocarcinomas, but was not detected on bronchial epithelial cells or pneumocytes in fetal or adult lung parenchyma (Matsuda *et al.*, 1993). This finding suggests that aberrant c-*kit* expression by lung cancer cells may be involved in the etiology of their malignant growth (Matsuda *et al.*, 1993). The possible involvement of a c-*kit*-dependent autocrine or paracrine growth loop in SCLC has been suggested by the fact that some SCLC tumors and tumor cell lines coexpress *SCF* and c-*kit* mRNAs (Hibi *et al.*, 1991; Turner *et al.*, 1992) and by the presence of SCF protein in the culture supernatant and on the cell surface of the H69 small cell lung carcinoma cell line (Turner *et al.*, 1992). Moreover, in a preliminary report, Armstrong *et al.* (1993) found that 70% of SCLC cell lines coexpressed c-*kit* and *SCF*, and that transfection of a c-*kit* expression vector into an SCLC cell line that

expressed *SCF* but not c-*kit* abolished the requirement of the cell line for serum for *in vitro* growth.

H. Breast Cancer

SCFR protein has been detected in normal mammary ductal cells, indicating that the c-*kit* signaling pathway may be involved in the normal development, differentiation, or function of these cells (Matsuda *et al.*, 1993). On the other hand, only a small fraction of breast cancers examined (2/10) express c-*kit*, and these do so at relatively low levels (Matsuda *et al.*, 1993). Too few data are available to determine whether reduced c-*kit* expression is functionally important in the growth or metastasis of breast cancer, e.g., by allowing the tumor cells to escape SCFR-dependent interactions with stromal cells that may regulate cell growth (Matsuda *et al.*, 1993). On the other hand, the Du4475 breast carcinoma cell line was recently reported to coexpress c-*kit* and SCF and to secrete SCR protein into the culture medium (Turner *et al.*, 1992).

XV. SCF as a Therapeutic Agent

As with any cytokine, the development of clinical applications for SCF will depend on both a thorough understanding of the basic biology of the cytokine and the appropriate selection of clinical settings for its use. Much of the clinical interest in SCF focuses on its effects on primitive hematopoietic cells, because this population can be impaired either as a result of a variety of primary hematological disorders or as a result of treatment of malignancies with radiation and/or chemotherapy.

Two colony-stimulating factors, G-CSF and GM-CSF, have already been approved for treating bone marrow damage induced by chemotherapy or in the setting of bone marrow transplantation. However, there are several important differences between SCF and G-CSF or GM-CSF. First, except for mast cells, SCF is not as potent a differentiation factor as G-CSF or GM-CSF. Second, the differentiation of the primitive hematopoietic cells, which represent the targets of SCF, appears to be highly regulated, and the expansion of such cells by SCF administration *in vivo* in the absence of other differentiation stimuli generally will not result in striking elevations of the peripheral leukocyte count.

For example, G-CSF can expand neutrophil precursors and induce their differentiation into mature neutrophils, which retain receptors for G-CSF. But most hematopoietic cells progressively lose the SCFR

on further differentiation. Accordingly, SCF administration *in vivo* can strikingly expand primitive hematopoietic cells but this is not reflected in increases of the same magnitude in the differentiated progeny. Thus, in nonhuman primates, *in vivo* administration of SCF produced a 300- to 1000-fold increase in primitive hematopoietic progenitors in the bone marrow and the peripheral blood, but only a 3- to 5-fold increase in most differentiated leukocytes in the circulation (Andrews *et al.*, 1992). Moreover, when administration of SCF was discontinued, numbers of both primitive progenitors and peripheral blood leukocytes rapidly returned to normal (R. G. Andrews *et al.*, 1991, 1992).

It may seem reasonable to expect that administering SCF together with other growth factors that induce differentiation may result in enhancement of the generation of differentiated progeny of the primitive cells that are expanded by SCF alone. Yet certain data indicate that this may not be so. For example, Sl/Sl^d mice have a macrocytic anemia and reduced numbers of CFU-S (primitive hematopoietic cells), yet their peripheral blood granulocyte and platelet counts are relatively normal. This interesting phenomenon probably reflects, at least in part, the occurrence of compensatory mechanisms that amplify numbers of intermediate committed progenitors. Thus, EPO levels in these mice are ~1000 times normal (Russell, 1979). When SCF was administered to Sl/Sl^d mice, there was an initial increase in neutrophils and platelets, followed by an increase in hematocrit (Zsebo *et al.*, 1990b; Bodine *et al.*, 1992). However, after 2 weeks, the neutrophil and platelet counts returned to normal. The hematocrit remained normal, and numbers of CFU-S remained elevated, for as long as SCF was administered. When the same amount of SCF was administered to normal mice, the only major effects were increases in primitive hematopoietic precursors and mast cells (Molineux *et al.*, 1992; Tsai *et al.*, 1991a).

These findings suggest that, in mice, the primitive precursor(s) that respond to SCF can differentiate within specific hematopoietic lineages, such as toward red cells, but that this primarily happens when that lineage is defective. Under such circumstances, the relevant later-acting factors, such as EPO, may be elevated as a result of the deficiency in the target population. The findings also suggest that numbers of differentiated hematopoietic cells in the circulation are highly regulated, and may not be expanded strikingly by measures that expand specific populations of precursor cells. Finally, the data demonstrate that the mast cell lineage can respond as a result of the *in vivo* administration of SCF alone, probably because the size of mast cell populations in normal or diseased tissues is importantly regulated by levels of endogenous SCF (see Section X,A).

Based on currently available information, the most appropriate clinical settings for SCF are those in which very immature hematopoietic precursors are damaged or defective, or production of SCF by stromal cells is impaired. The most obvious of these settings is aplastic anemia, because the patients have deficiencies in very primitive hematopoietic cells and also have elevated levels of late-acting cytokines such as EPO and thrombopoietin. *In vitro* studies employing bone marrow samples from patients with aplastic anemia who exhibited partial clinical improvement secondary to antilymphocyte globulin treatment indicate that addition of SCF enhanced by >10-fold the proliferation of bone marrow cells maintained in a combination of EPO, IL-3, GM-CSF, and G-CSF, but that none of the patients' cells responded to SCF with a sensitivity comparable to that of normal bone marrow cells (Wodnar-Filipowicz *et al.*, 1992). Although the basis for this observation is not yet clear, it is compatible with the possibility that bone marrow cells in patients with aplastic anemia exhibit abnormalities of the SCF/SCFR signaling pathway. *In vitro* studies also suggest that SCF may be of benefit in some patients with constitutional pure red cell aplasia, or Diamond–Blackfan anemia (Abkowitz *et al.*, 1991; Olivieri *et al.*, 1991), and in patients with immunodeficiency virus-related cytopenias (Miles *et al.*, 1991), and perhaps to stimulate normal hematopoiesis in patients with AML or myelodysplastic syndromes (Goselink *et al.*, 1992). SCF also can significantly increase (by 66%) the proportion of fetal hemoglobin in progenitor cells derived from the peripheral blood of patients with sickle cell disease (Miller *et al.*, 1992). The extent to which these *in vitro* effects will be observed *in vivo*, and assessment of whether such effects will be of benefit in these conditions, await the results of clinical trials.

Another appropriate target group is bone marrow transplant recipients, who develop bone marrow stromal cell damage secondary to radiation treatment and who exhibit long-term depression of levels of circulating SCF after the conditioning treatments (K. M. Zsebo *et al.*, unpublished data). High dose multiple-cycle chemotherapy patients may benefit as well. However, it is less clear that patients who receive standard dose chemotherapy will benefit from SCF, since these patients exhibit defects in relatively mature progenitors rather than primitive hematopoietic cells.

One of the most exciting potential uses of SCF could be to expand primitive precursors and to mobilize them into the peripheral blood, in order to harvest and store these cells *prior* to treatments that damage hematopoietic or stromal cells. The cells then could be infused into the patients after each cycle of radiation or chemotherapy. Alternatively,

SCF might be used in conjunction with other hematopoietic growth factors to expand hematopoietic progenitor cells *in vitro* (Brugger *et al.*, 1993). Another promising application, supported by the demonstration that SCF in combination with other hematopoietic growth factors can enhance retroviral vector-mediated gene transfer into primitive hematopoietic cells (Nolta *et al.*, 1992), would be to employ SCF in gene therapy protocols (Lyman and Williams, 1992).

Phase I studies of SCF, conducted in hematologically normal cancer patients, indicate that SCF can expand numbers of circulating hematopoietic progenitor cells in both breast cancer (Demetri *et al.*, 1993a,b) and non-small cell lung cancer (Kurtzberg *et al.*, 1993) patients. In contrast, SCF had modest (Orazi *et al.*, 1993; Tong *et al.*, 1993) or little or no (Demetri *et al.*, 1993a; Kurtzberg *et al.*, 1993) effect on numbers of hematopoietic progenitor cells in the bone marrow of these patients. In one of these phase I studies, Costa *et al.* (1993) showed that the patients also developed a modest mast cell hyperplasia, with densities of mast cells in skin not directly injected with SCF increasing to levels ~70% higher than those at the start of treatment. Some of the patients also developed signs or symptoms that may have reflected SCF-dependent mast cell activation (Demetri *et al.*, 1993b). Although the multiple actions of SCF on mast cells always should be kept in mind during clinical uses of this cytokine, studies in both mice (S. J. Galli *et al.*, unpublished data) and nonhuman primates (Galli *et al.*, 1993) indicate that SCF-induced mast cell hyperplasia is rapidly reversible on discontinuation of the agent (see Section X,A). Moreover, the patients in phase I trials of SCF who developed clinical signs or symptoms of mast cell activation responded well to standard therapy for such problems (Demetri *et al.*, 1993b). Taken together, these findings suggest that SCF-dependent effects on mast cells will be clinically manageable.

Initial clinical studies of SCF have focused on its actions on hematopoietic cells. However, its effects on other lineages also may have therapeutic uses. For example, its effects on germ cells (Section XII) raise the possibility that SCF may be useful in certain infertility syndromes.

XVI. Concluding Remarks

Many years ago, alert observers noticed among thousands of laboratory mice a few individuals that, unlike their littermates, exhibited areas of white spotting on their fur (reviewed in Russell, 1979; Silvers, 1979a). No one could have predicted then that an effort to understand

the basis for these abnormalities would ultimately contribute to the characterization of a receptor (SCFR) and a corresponding ligand (SCF) that are critical not only to the migration and development of melanocytes, but to hematopoiesis, gametogenesis, mast cell development, and, perhaps, aspects of central nervous system development or function. Nor could anyone have foretold then that this receptor and ligand would be shown to regulate the development of multiple distinct cellular lineages not only in mice, but also in humans and other primates, or that the SCFR and SCF would be found to influence the secretory function of cells bearing this receptor, as well as their development.

Investigation of the effects of SCF on a single cell type, the mast cell, has produced the most complete picture of the spectrum of biological processes that can be regulated by interactions between the SCFR and SCF. This work shows that SCF critically regulates the migration and survival of mast cell precursors and mast cell adherence, promotes the proliferation of both immature and mature mast cells, enhances mast cell maturation, directly induces secretion of mast cell mediators, and regulates the extent of mediator release in mast cells activated by IgE-dependent mechanisms. Indeed, SCF may well prove to be one of the most important of the factors that influence mast cell numbers, phenotype, and function in both health and disease.

SCF also has a major role in lymphohematopoiesis. Notably, many of the actions of SCF in hematopoiesis and in lymphocyte development, including effects on the survival and proliferation of early hematopoietic cells, as well as actions that augment the maturation/differentiation of diverse lineages of hematopoietic or lymphoid cells, are expressed in large part through synergistic interactions with other growth factors. These properties indicate that SCF, when used alone or perhaps in conjunction with other growth factors, may have a broad spectrum of clinical uses in settings where promoting the *in vitro* or *in vivo* survival or expansion of early hematopoietic or lymphoid cells, and augmenting the production of the more differentiated progeny of these cells, represent therapeutic goals. In analogy to its effects on mast cell function, SCF may also have the additional clinical value of promoting the effector function of certain myeloid or lymphoid lineages. Indeed, evidence already has been published indicating that this cytokine may enhance the cytolytic function of certain lymphoid effector cells. The ability of SCF to enhance retroviral-mediated gene transfer into primitive hematopoietic cells (Nolta *et al.*, 1992) points to additional potential roles for this cytokine in gene therapy protocols.

As with any therapeutic agent, maximizing the net clinical benefit of

SCF will require that the agent be used in a manner that optimizes the desired effects while minimizing unwanted actions. Preliminary data indicate that the administration of rhSCF can significantly expand numbers of early hematopoietic cells in humans, particularly in the peripheral blood (Demetri *et al.*, 1993a,b; Kurtzberg *et al.*, 1993; Orazi *et al.*, 1993; Tong *et al.*, 1993). rhSCF-treated subjects also exhibit mast cell hyperplasia (Costa *et al.*, 1993) and occasional signs or symptoms that may reflect the agent's ability to induce or augment mast cell activation (Demetri *et al.*, 1993b). However, taken together, the early evidence from human trials of SCF indicates that its effects on early hematopoietic cells are promising and that any untoward effects related to actions on mast cells will be manageable.

Beyond its potential value in clinical applications, SCF is of interest because of its many effects in normal gametogenesis and melanocyte development, the unique opportunity provided by *W*/c-*kit* and *Sl*/*SCF* mutant animals for understanding the biology and molecular biology of an RTK and its cognate ligand, the possibility that abnormalities in SCF and/or SCFR may be involved in the pathogenesis of certain neoplasms, the interest in interactions between SCF and its receptor as a model for understanding the roles of protooncogenes in mammalian development, and the questions concerning the specific roles of SCF and the SCFR in the development or function of tissues, such as the central nervous system, which do not exhibit obvious phenotypic abnormalities in *W*/c-*kit* or *Sl*/*SCF* mutant animals. In short, much additional work will be required to elucidate fully the roles of SCF and its receptor in mammalian biology, and to understand these processes at the molecular level.

The excitement generated by the identification of the SCFR as the product of the c-*kit* gene, and of SCF as the product encoded at the mouse *Sl* locus, may have created the impression that the molecular basis for the pleiotropic effects of mutations at these loci had finally been revealed. But the identification of the genes encoded at *W* and *Sl* and the availability of the corresponding products have resulted in the generation of new information showing that the biology of SCF and its receptor is even more complex than had been previously suspected. Thus, as is usually the case in science, the answers to one set of questions have raised new questions, which are related to but different from the first, and quite interesting in their own right. On the other hand, the tools are now in hand to pursue aggressively, and ultimately to resolve, many of these issues. In a very different context, Winston Churchill (as quoted in Gilbert, 1986) expressed sentiments that seems very appropriate to describe this stage in our current understanding of

the biology of SCF and SCFR: "Now this is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning."

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Interleukin-8 and Related Chemotactic Cytokines—CXC and CC Chemokines

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I. Introduction

Interleukin-8 (IL-8) is the best known member of a new class of cytokines that are widely studied because of their ability to attract and activate leukocytes, and their potential role as mediators of inflammation. All of these cytokines have four conserved cysteines, and two subfamilies can be distinguished according to the position of the first two cysteines, which either are separated by one amino acid (CXC proteins) or are adjacent (CC proteins). The members of the two subfamilies differ in their target cell selectivity and the chromosomal location of their genes (chromosome 4 for the CXC proteins and chromosome 17 for the CC proteins). At a meeting where most groups working in this field were represented, the term *chemokines* was proposed for this novel class of proteins (1). Several reviews on CXC and CC chemokines have appeared since 1989 (1a–9), and they may be consulted for aspects that are not covered in the present review, which focuses on human chemokines.

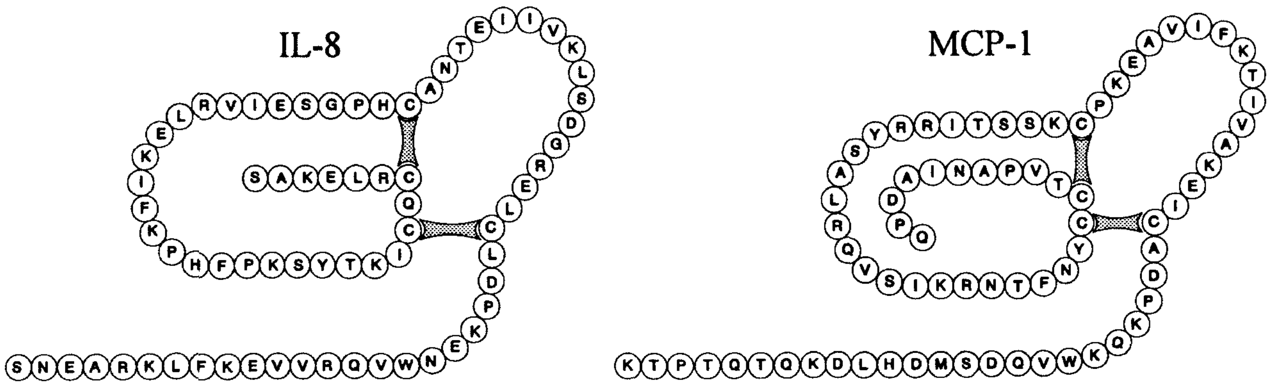
II. Structures and Chemical Properties

A. IL-8 and Other CXC Chemokines

Platelet factor 4 (PF4) was the first CXC protein to be characterized. Its sequence was reported in 1977 (10–13), 10 years before the discovery of IL-8 (14–16) (Fig. 1). PF4 is stored in the α-granules of blood platelets, which contain two other CXC proteins, platelet basic protein (PBP) and its N-terminal truncation derivative, connective tissue-activating peptide III (CTAP-III) (17, 18). β-Thromboglobulin, a further truncation product of PBP, was studied extensively by Begg *et al.* (19), who characterized the two disulfide bonds linking the first cysteine to the third, and the second cysteine to the fourth.

IL-8 was originally isolated from the culture supernatants of stimulated human blood monocytes and was identified as a protein of 72 amino acids with a molecular weight of 8383 (15, 16). The open reading frame of the IL-8 cDNA codes for 99 amino acids (14, 20), and the

		CXC		C		C	
IL-8 (77)	AVLPRSAKELR	CQC	IKTYSKPFHPKFIKELRVIESGPH	C	ANTEIIVKLSD-GREL	C	LDPKENWVQRVVEKFLKRAENS
GRO α (73)	ASVATELR	CQC	LQT-LQGIHPKNIQSVNVKSPGPH	C	AQTEVIATLKN-GRKA	C	LNPASPIVKKIIEKMLNSDKSN
GRO β (73)	APLATELR	CQC	LQT-LQGIHLKNIQSVKVKSPGPH	C	AQTEVIATLKN-GQKA	C	LNPASPMVKKIIEKMLKNGKSN
GRO γ (73)	ASVVTELR	CQC	LQT-LQGIHLKNIQSVNVRSPGPH	C	AQTEVIATLKN-GKKA	C	LNPASPMVQKIIEKILNKGSTN
NAP-2 (70)	AELR	CMC	IKT-TSGIHPKNIQSLEVIGKGTH	C	NQVEVIATLKD-GRKI	C	LDPDAPRIKKIVQKKLAGDESAD
ENA-78 (78)	AGPAAAVLRELR	CVC	LQT-TQGVHPKMISNLQVFAIGPQ	C	SKVEVVASLKN-GKEI	C	LDPEAPFLKKVIQKILDGGNKEN
PF4 (70)	EAEEDGDLQ	CLC	VKT-TSQVRPRHITSLEVIKAGPH	C	PTAQLIATLKN-GRKI	C	LDLQAPLYKKIIKKLLES
IP-10 (77)	VPLSRTVR	CTC	ISISNQPVNPRSLEKLEIIPASQF	C	PRVEIIATMKKKGEKR	C	LNPESKAIKNLLKAVSKEMSKRSP
GCP-2	GPVSAVLTELR	CTC	LRVTLR.....				
		C–C		C		C	
MCP-1 (76)	QPDAINAPVT	C–C	YNFTNRKISVQRLASY-RRITSSK	C	PKEAVIFK-TIVAKEI	C	ADPKQKWVQDSMDHLDKQTQTPKT
HC14 (76)	QPDSVSIPIT	C–C	FNVINRKIPIQRLESY-TRITNIQ	C	PKEAVIFK-TKRGKEV	C	ADPKERWVRDSMKHLDQIFQNLKP
MCP-3 (76)	QPVGINTSTT	C–C	YRFINKKIPKQRLESY-RRTTSSH	C	PREAVIFK-TKLDKEI	C	ADPTQKWVQDFMKHLDKKTQTPKL
I-309 (74)	SKSMQVPFS-R	C–C	FSFAEQEIPLRAILCY--RNTSSI	C	SNEGLIFK-LKRGKEA	C	ALDTVGWVQRHRKMLRHCPSKRK
MIP-1α, LD78 (70)	ASLAADTPTA	C–C	FSYTSRQIPQNFIADY--FETSSQ	C	SKPGVIFL-TKRSRQV	C	ADPSEEWVQKYVSDLELSA
MIP-1β, Act-2 (69)	APMGSDPPTA	C–C	FSYTARKLPRNFVVDY--YETSSL	C	SQPAVVFQ-TKRSKQV	C	ADPSESWVQEYVYDLELN
RANTES (68)	SPYSSDT-TP	C–C	FAYIARPLPRAHIKEY--FYTSGK	C	SNPAVVFV-TRKNRQV	C	ANPEKKWVREYINSLEMS



protein is secreted after cleavage of a 20-amino acid signal sequence (21). The mature form is processed further at the N terminus, yielding several biologically active truncation analogs (21–23). The processing is due to proteases released by the cells, which generate IL-8, and the occurrence of N-terminal variants depends on the cell type and culture conditions. Of the two major forms, the 72-amino acid protein (SAKELRC· · ·) predominates in cultures of monocytes and macrophages (22), and the 77-amino acid protein (AVLPRSAKELRC· · ·) predominates in cultures of tissue cells, e.g., endothelial cells (24, 25) and fibroblasts (26, 27).

The three-dimensional structure of IL-8 has been studied by nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography. In concentrated solution and on crystallization, IL-8 is present as a dimer (28, 29). The monomer has a short, conformationally flexible N-terminal domain followed by three antiparallel β-strands connected by loops, and a prominent α-helix extending from residue 57 to the C terminus. The dimer is stabilized by six hydrogen bonds between the first β-strands of the partner molecules (residues 23–29) and by side chain interactions. The dimer thus consists of two antiparallel α-helices lying on top of a six-stranded antiparallel β-sheet. A similar structure has been established for PF4 (30) and CTAP-III (31). PF4 and CTAP-III were reported to aggregate into dimers and tetramers, but NMR studies indicate that the equilibrium favors the monomeric state (31, 32).

CXC chemokines with biological activities similar to those of IL-8 were discovered in rapid succession. Neutrophil-activating protein-2 (NAP-2) was identified in the conditioned media of monocytes cultured in the presence of blood platelets, and found to arise from the N-terminal processing of the two platelet α-granule storage proteins,

FIG. 1. Amino acid sequences and two-loop structure of human chemokines. CXC and CC chemokines are aligned according to their cysteines (shaded areas). The mature forms are deduced from protein or cDNA sequence data for IL-8 (22), GROα (37, 82), GROβ and GROγ (41), NAP-2 (34), ENA-78 (45), PF4 (10–13), IP10 (49), MCP-1 (58, 59), HC14 (65), MCP-3 (67, 67a), and RANTES (64). The mature form of I-309 has not been reported (62) and was chosen arbitrarily. Several isoforms have been reported for human MIP-1α and MIP-1β (7, 9), and we have chosen to present those described initially for LD78 (51) and Act-2 (52). The sequence of GCP-2 is only partially known (46). The number of amino acids of the sequence shown is indicated in parentheses after the name of the protein. Amino acids in one-letter code are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

PBP and CTAP-III, by monocyte-derived proteases (33–35). In these experiments three NAP-2 variants with three, four, and five additional N-terminal residues were also detected (35). GROα was originally described as a melanoma growth stimulatory activity (MGSA) (36, 37) and was subsequently shown to activate human neutrophils (38–40). Two additional *GRO* gene products (GROβ and GROγ) were later discovered (41–43). The three GRO proteins have about 90% sequence identity and similar neutrophil-activating properties (44). An epithelial cell-derived neutrophil-activating protein, ENA-78, was identified as a product of type II alveolar cells (45), and a novel CXC chemokine, granulocyte chemotactic protein-2 (GCP-2), was isolated from the conditioned medium of human osteosarcoma cells (46). Both chemokines activate and attract human neutrophils, but are less potent than IL-8. Of all known inducible CXC proteins, only one is inactive toward neutrophils, the interferonγ (IFN-γ)-inducible protein IP10 (47), which was first described by Luster *et al.* (48, 49), and is expressed in delayed-type hypersensitivity reactions in human skin (50).

IL-8 shares with the other CXC proteins sequence identities ranging from 24 to 46% (Fig. 1, Table I). The degree of overall sequence

TABLE I
AMINO ACID IDENTITIES AMONG CXC AND CC CHEMOKINES[a]

	GROα	GROβ	GROγ	NAP-2	ENA-78	PF4	IP10
IL-8[b]	43	42	40	46	33	39	24
GROα		88	85	60	52	46	29
GROβ			84	56	52	41	30
GROγ				54	52	41	25
NAP-2					53	57	33
ENA-78						44	33
PF4							37
	HC14	MCP-3	MIP-1α	MIP-1β	RANTES	I-309	
MCP-1	62	71	39	36	29	31	
HC14		60	40	39	35	34	
MCP-3			30	35	25	40	
MIP-1α				68	47	34	
MIP-1β					52	32	
RANTES						28	

[a] Numbers refer to percentage amino acid identity of the respective pair of peptides. Sequence alignments were carried out with the program PALIGN (414) using the Unitary Matrix (415).

[b] The 72-amino-acid form of IL-8 (22) was used for the alignment. All sequences are listed in Fig. 1.

identity with IL-8 does not necessarily correlate with the biological activity of the proteins. The amino acid identity is significantly lower between IL-8 and ENA-78, which are both active on neutrophils, than between IL-8 and the inactive analog PF4. A major determinant of activity and potency toward neutrophils is the N-terminal domain, which must be short and contain the sequence Glu-Leu-Arg (ELR) preceding the first cysteine (see Section VI,D,2). PBP and CTAP-III, which contain the ELR motif, are inactive, but are converted into the neutrophil chemoattractant NAP-2 by N-terminal truncation (35).

B. CC Chemokines

The first CC chemokine was identified after cloning by differential hybridization from human tonsillar lymphocytes, and was termed LD78 (51). Several cDNA isoforms of a closely related chemokine, Act-2 (52), were later identified (9) and two similar proteins, MIP-1α and MIP-1β, were purified from the culture medium of murine macrophages stimulated with lipopolysaccharide (LPS) (53) and subsequently cloned (54). The murine proteins are considered as the homologues of LD78 and Act-2 because of their amino acid identity of more than 70%, and the terms human MIP-1α and MIP-1β are commonly used instead of LD78 and Act-2.

The best characterized CC chemokine is MCP-1. It was purified from culture supernatants of blood mononuclear cells (55), as well as glioma (56) and myelomonocytic (57) cell lines, and was cloned from different sources (58–61). Other CC chemokines, I-309 (62, 63), RANTES (64), and HC14 (65), were purified or cloned as products of activated T cells. HC14, also termed MCP-2, was purified along with MCP-3 from cultures of osteosarcoma cells (66). MCP-3 has recently been cloned (67, 67a).

As shown in Fig. 1 and Table I, the CC and CXC chemokines are similar in size and have an overall structure that is characterized by the two intrachain disulfide bonds, short N-terminal and long C-terminal sequences. The cDNA clones encode proteins of 92 to 99 amino acids with characteristic putative leader sequences of 20–25 amino acids. The sequence identities of the mature CC chemokines with MCP-1 are between 29 and 71%. Although they all act on monocytes, with the possible exception of MIP-1β, CC chemokines lack a common N-terminal sequence, in analogy to the ELR motif of CXC chemokines. I-309 is the only chemokine with six instead of four cysteines (62). MCP-1 occurs in two electrophoretically distinct forms that differ in

glycosylation (55, 56). Its carbohydrate moiety has been partially characterized (68–70).

The three-dimensional structure of MCP-1 has been modeled on the basis of the NMR-derived structure of IL-8. Although the sequence identity between the two chemokines is low, the modeling yields a high degree of folding analogy (71, 72), suggesting that CXC and CC chemokines may have a similar configuration and behavior in solution.

C. Proteolytic Processing and Inactivation

IL-8 resists most treatments that denature and inactivate proteins, such as heat, low and high pH, and exposure to detergents or organic solvents (73). It is also resistant to plasma peptidases and is inactivated only slowly, at 37°C, by cathepsin G, elastase, and proteinase 3, with half-lives of approximately 2, 4, and 17 hours, respectively. NAP-2 and GROα are more susceptible to proteinase 3 (half-lives 0.2 and 2 hours), but more resistant to elastase and cathepsin G (half-lives 7–24 hours) (73a). These unusual properties are presumably due to the conformation of the native protein. Reduction of the disulfide bonds leads to unfolding and inactivation, but IL-8 rapidly regains its biologically active form on dialysis. As judged by electrophoretic analysis of the cleavage products, IL-8 becomes highly susceptible to proteases when refolding is prevented by alkylation of the cysteines, suggesting that resistance to proteolysis is largely dependent on its tertiary structure (73a).

IL-8 (21–23) and other CXC and CC chemokines (35, 74–76) are readily processed at the N terminus, as shown by the occurrence of several truncation variants. According to the three-dimensional NMR structure, the N-terminal sequence preceding the first cysteine is exposed and conformationally disordered, and it is not surprising that it is susceptible to proteases. The virtual absence of the 79-amino-acid form of IL-8 (21) and the frequent occurrence of variants with 72, 70, and 69 amino acids in biological fluids (21–23, 25, 26) suggest that processing continues until the N-terminal sequence becomes practically unaccessible to proteases. Because the potency increases with progressive cleavage (35, 74, 77) (see Section VI,D,2), N-terminal processing could represent a mechanism for the amplification of chemokine activity.

Conversion of the 77- to the 72-amino-acid form of IL-8 is observed in the presence of thrombin (25) or a neutrophil granule extract (73a). Proteinase 3, a serine protease that is localized in the azurophilic granules (78, 79), converts the 77- to the 70-amino-acid form of IL-8 (73a). Cathepsin G cleaves preferentially the bond between Tyr-15 and Ala-16 of CTAP-III, yielding NAP-2. No conversion of CTAP-III is

obtained, however, with thrombin, plasmin, and serine proteases from the granules of cytolytic T lymphocytes (76)

III. Chemokine Genes

A. Organization and Chromosomal Localization

The genes for the CXC chemokines, i.e., IL-8 (80, 81), GROα, GROβ, and GROγ (37, 41, 82), IP10 (83–85), PF4 (86, 87) and PBP (88, 89), the precursor of NAP-2, are colocalized on human chromosome 4, q12–21. The PF4 and PBP genes (86) consist of three exons and two introns, whereas the other CXC chemokine genes have an additional intron and exon (Fig. 2). The similarity of the splice junctions and of the sizes of the introns and exons, however, suggests that they all stem from a common ancestral gene. The genes for the CC chemokines, i.e., MCP-1 (90–92), MIP-1α, MIP-1β (93, 94), I-309 (63), and RANTES (95), have a three-exon/two-intron structure like the PF4 gene, and are clustered on chromosome 17, q11–21 (Fig. 2).

It is conceivable that all chemokines share the same ancestral gene with a three-exon/two-intron structure, and that CC chemokine genes have undergone chromosomal translocation, whereas the genes for IL-8, GRO, PF4, PBP, and IP10 may have arisen locally through gene duplication. All three human *GRO* genes, which share over 80% nucleotide sequence identity, map to a single band, 4q21. A single location, on chromosome 17, is also observed for the genes of human MIP-1α

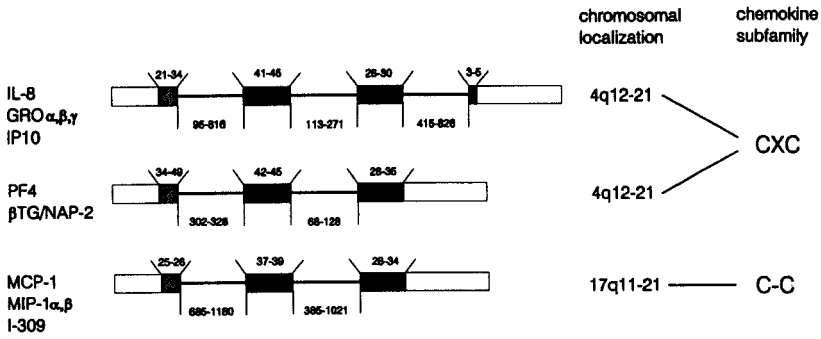


FIG. 2. Organization and chromosomal localization of chemokine genes. Exons are represented by boxes, and introns by lines. The coding regions for the signal peptides and the mature proteins are hatched and black, respectively, and the 5′ and 3′ untranslated regions are white. The number of amino acids corresponding to the coding regions is indicated above the boxes, and the length of the introns (in base pairs) is given below the lines.

and MIP-1β, which are more than 65% identical (93, 94). These findings suggest that the relative distances within the chromosome are inversely related to the structural similarity, which would be in keeping with an origin by gene duplication (41).

B. Regulation of Gene Expression

Matsushima and colleagues have isolated and sequenced genomic fragments comprising the entire human IL-8 gene (80). The region between −425 and −70 bp preceding the first exon carries putative binding sites for several transcription regulatory elements, including NF-κB, NF-IL-6, an octamer-binding motif, AP-1, glucocorticoid receptor, hepatocyte nuclear factor-1, and interferon regulatory factor-1. As shown by chloramphenicol acetyltransferase expression experiments, a relatively small region (−94 to −71 bp), which includes the presumed NF-κB and NF-IL-6 binding sequences, and the −50 to −1 bp region with the TATA box contain the minimal enhancer elements for mRNA induction by stimulation with IL-1, tumor necrosis factor (TNF), phytohemagglutinin (PHA), or hepatitis B virus protein X (96, 97). Gel retardation assays show the potential involvement of NF-IL-6- and NF-κB-like factors as cis-acting elements in IL-8 mRNA expression. Activation of the IL-8 gene in human monocytes by IL-1, TNF, or PHA may also depend on phosphorylations by serine kinase(s) (98).

Several agents affect cytokine-induced IL-8 production, including 1,25-dihydroxycholecalciferol (99), glucocorticoids (98, 100–102), IL-4 (103), and IFN-γ (104, 105). Some of these effects appear to be cell specific. IL-4, for instance, inhibits IL-1-, TNF-, or LPS-induced IL-8 mRNA expression in monocytes, but not in fibroblasts, epithelial, or endothelial cells (103, 104, 106). IFN-γ synergizes with TNF as inducer of IL-8 expression in keratinocytes (107), but inhibits IL-8 mRNA expression in thymic epithelial cells and neutrophils (104, 105). IL-8 mRNA expression after stimulation with IL-1 or TNF is rapid and results at least in part from transcriptional activation, as shown by nuclear run-off assays (108–110). Cycloheximide enhances expression, presumably by inhibiting the synthesis of negative regulatory elements (nucleases, repressors) that are coinduced (109, 110). The stability of IL-8 mRNA may be influenced by the RNA instability element, AUUUA, found in the 3′ untranslated region (20, 111).

The 5′ untranslated regions of the genes for GROα, GROβ, and GROγ are highly homologous up to position −136 and contain binding motifs for NF-κB and AP-3, and a TATA box (41, 112, 113). The sequences diverge further upstream and contain additional binding motifs for nuclear factors. Chloramphenicol acetyltransferase expression

studies show that the region between −65 and −84, which contains the NF-κB binding motif, is required for expression after stimulation with IL-1 and TNF (112). Gel retardation assays document the induction of a factor that binds to this region in IL-1- or TNF-stimulated fibroblasts (114). As for IL-8, expression of GRO transcripts is rapid and sustained. Each of the three GRO genes contains in the 3′ region up to 11 AUUUA mRNA instability elements that may influence RNA degradation (115, 116). Superinduction by cycloheximide is observed for the GRO genes as for the IL-8 gene (112). A novel seven-nucleotide motif, found in the 3′ untranslated regions of many "immediate-early genes," including those for the GRO proteins, is considered as a response element in the growth-related gene expression (117).

Much less information is available on the regulation of CC chemokine genes. As observed for IL-8, IL-1 and TNF are major stimuli of MCP-1 expression in a wide variety of cells. It is interesting, however, that the upstream region of the MCP-1 gene in humans, mice, and rats differs from the corresponding region of the IL-8 gene (61, 118, 119), although potential binding sites for nuclear factors, including NF-κB, NF-IL-6, AP-1, and AP-2, have been identified (119). The role of these elements for activation of the MCP-1 gene, however, remains to be established.

IV. Cellular Sources of CXC and CC Proteins

A. Monocytes, Macrophages, and Other Leukocytes

Monocytes produce many cytokines and growth factors, and are a rich source of CXC and CC chemokines. Cytokine secretion is usually associated with *de novo* synthesis. *Escherichia coli* endotoxin (LPS) is one of the most powerful stimuli, and it is not surprising that IL-8 was discovered in cultures of monocytes exposed to LPS (15, 16, 120–122). It was soon realized, however, that these cells express and secrete IL-8 in response to a multitude of proinflammatory agents, such as IL-1α (123), IL-1β (103), TNFα (20), IL-7 (124), GM-CSF and IL-3 (125), concanavalin A, phytohemagglutinin (73, 122), zymosan (126, 127), immune complexes (127), and bacteria (128–131). Even adherence to plastic (132, 133) or changes in oxygen partial pressure (134) are sufficient to trigger production of IL-8. In the absence of a stimulus, as shown by monocytes in whole blood (135, 136) or after isolation under aseptic conditions (73, 132), IL-8 mRNA is virtually undetectable and no IL-8 is released.

IL-8 is produced by fetal monocytes (137) and mononuclear cells isolated from the bone marrow and stimulated with LPS (127, 138).

Tissue macrophages release large amounts of IL-8 on exposure to LPS (139, 140), IL-1β, TNFα (141), or zymosan (126) or after infection with respiratory syncytial virus (142). Macrophages from lavage and exudate fluids of patients with inflammatory and proliferative diseases, e.g., idiopathic pulmonary fibrosis (143) or rheumatoid arthritis (127, 144–146), constitutively express high levels of IL-8 mRNA and release larger amounts of this chemokine compared to blood monocytes from the same individuals. Mononuclear cells from inflammatory effusions are also more responsive to stimulation with LPS, IL-1β, TNFα, concanavalin A, zymosan, and immune complexes (127, 146). GROα, GROβ, and GROγ are expressed and secreted by monocytes and macrophages after stimulation with LPS (40–42) or on adherence (41). IP10 expression is induced by IFNγ (48, 49), and its mRNA is found in humans in blood monocytes and alveolar macrophages after treatment with IFN-γ by subcutaneous injection or administration as aerosol (147).

MCP-1 is a major product of monocytes. Its expression is stimulated by phytohemagglutinin (55), LPS, IL-1β (58, 148), TNFα, GM-CSF, inactivated streptococci (148), and IFN-γ (65, 148), but production in the absence of added stimuli has also been reported (61, 149). The conditions for MCP-1 expression are not entirely clear since LPS was reported to be stimulatory (58, 148), inhibitory (61), or ineffective (139), and induction by IFN-γ has not been consistently observed (61). HC14 (MCP-2), a chemokine that is closely related to MCP-1, is expressed in parallel with MCP-1 in monocytes exposed to IFN-γ (65). Marked MCP-1 expression is observed in alveolar macrophages from patients with idiopathic pulmonary fibrosis (150) and in macrophage-rich atherosclerotic lesions (151, 152). Several stimuli, such as phytohemagglutinin, LPS, phorbol myristate acetate (PMA), IL-7, and adherence to various surfaces, including endothelial cells, also induce MIP-1β (Act-2) production in blood monocytes (52, 133, 153).

Lymphocytes are rich sources of some CC chemokines, for example, RANTES (62, 64, 154), I-309 (62, 63), MIP-1α (62, 154, 155), and MIP-1β (52, 153, 155), but are considerably less important than mononuclear phagocytes as producers of CXC chemokines, as shown for IL-8 (154, 156–158).

Neutrophils produce IL-8 in response to a variety of stimuli, including LPS (159–161), IL-1β, TNFα, adherence (161), and GM-CSF (125). Of particular interest is the production and release of IL-8 after stimulation with chemoattractants such as fMet-Leu-Phe, C5a, PAF, and LTB_4 (161, 162), and during phagocytosis (159), which suggests that neutrophils may be able to stimulate their own recruitment. The effect of fMet-Leu-Phe, C5a, and PAF is markedly increased in the presence

of LPS (161). The induction of IL-8 by fMet-Leu-Phe is prevented by pretreatment with *Bordetella pertussis* toxin, suggesting that chemokine gene expression and chemotaxis are triggered through the fMet-Leu-Phe receptor (162). Pretreatment of neutrophils with IFN-γ (102, 105) or dexamethasone (102) inhibits the expression and release of IL-8 by neutrophils stimulated with TNF, LPS, fMet-Leu-Phe, or phagocytosis. Neutrophils also produce GROα and GROβ on adherence to fibronectin (41) or exposure to LPS (42). No evidence for MCP-1 production by neutrophils has been found so far (160). However, it was shown that eosinophils release IL-8 after stimulation with the Ca^{2+} ionophore A23187, but not with TNFα or IL-1β (163).

B. Tissue Cells

The observation that IL-8 is not only a product of monocytes and macrophages, but is also generated by tissue cells, was very important for the understanding of the numerous potential implications of this chemokine in disease (1a). A wealth of information shows that several CXC and CC chemokines, like IL-8, are expressed in most tissues (Table II). IL-1 und TNF are the most common and powerful inducers of IL-8 (1a). They are frequently used to study chemokine expression, and well over 100 reports document their effectiveness in virtually all types of tissue cells.

IL-1α, IL-1β, TNFα, and LPS induce the production of IL-8 (164–166), GRO proteins (41, 167), and MCP-1 (60, 166, 168, 169) in human umbilical vein *endothelial cells*. Other endothelia presumably respond in a similar manner, as suggested by studies on MCP-1 production in lung and aortic tissues (150, 170). Cultured endothelial cells are also responsive to PMA and thrombin, as shown for the formation of GROα (167), and to IL-4 (171) and IFN-γ (169), which induce MCP-1. Experiments *in vitro* suggest that IL-8 released from stimulated endothelial cells facilitates the transendothelial migration of neutrophils (172). A similar situation is encountered with IL-1- or TNF-stimulated *epithelial cells,* which produce IL-8 (104, 173, 174), GROα, GROβ, and GROγ (41, 45, 175), ENA-78 (45), and MCP-1 (176, 177). Of interest are the reports showing expression of IL-8 by epithelial cells on virus infection (142, 178) and exposure to neutrophil elastase (179).

IL-1α, IL-1β, and TNFα are prominent stimuli of CXC and CC chemokine expression in *fibroblasts* of different origin. IL-8 production by these cells is also observed after exposure to double-stranded RNA poly(IC) (180), LPS from *Bacteroides intermedius* and *Bacteroides gingivalis* (131), and the antitumoral T cell-derived cytokine leukoregulin (181), as well as on virus infection (182, 183). Leuko-

TABLE II
CELLULAR SOURCES OF HUMAN CXC AND CC CHEMOKINES

Chemokine[a]	Source	Ref.
IL-8	Monocytes/macrophages	15, 16, 20, 73, 99, 103, 106, 120–130, 132, 134, 137, 138, 140–143, 148, 198, 199
	Neutrophils	102, 105, 125, 159–162
	T lymphocytes	154, 156–158
	Endothelial cells (HUVEC)	108, 123, 164–166, 169, 171, 416
	Epithelial cells	
	Cornea	417
	Kidney	418
	Liver	419
	Lung	45, 174, 178, 179, 420–422
	Retina	173
	Thymus	104
	Thyroid	423
	Fibroblasts	
	Gingiva	131, 424
	Lung	27, 200, 391
	Skin	26, 27, 123, 180–182, 203, 391, 425–427
	Astrocytes	428
	Chondrocytes	392–394
	Keratinocytes	107, 123, 381, 382, 427, 429
	Mesangial cells	202, 430–432
	Smooth muscle (vascular)	433
	Synovium	
	Fibroblasts	27, 145, 204, 391
	Macrophages	145
	Tumor cells	
	Astrocytoma	434
	Lung carcinoma	435
	Osteosarcoma	182
	Melanoma	279
	Renal carcinoma	191
	Fibrosarcoma	101
	Glioblastoma	434
GROα	Monocytes	40, 41
	Neutrophils	41
	Endothelial cells (HUVEC)	41, 167
	Epithelial cells	
	Lung	45
	Mammary	45, 175
	Fibroblasts	
	Lung	27, 391
	Mammary	41
	Skin	27, 37, 41, 114, 175, 391
	Keratinocytes	36
	Synovium (fibroblasts)	27, 391

TABLE II *Continued*

Chemokine[a]	Source	Ref.
	Tumor cells	
	Melanoma	36, 37, 190, 193
	Renal carcinoma	37
	Bladder carcinoma	41
GROβ	Monocytes	41, 42
	Neutrophils	41, 42
	Endothelial cells (HUVEC)	41
	Epithelial cells (mammary)	41
	Fibroblasts	
	Mammary	41
	Skin	41
GROγ	Monocytes	41
	Neutrophils	41
	Endothelial cells (HUVEC)	41
	Epithelial cells	
	Lung	45
	Mammary	41
	Fibroblasts	
	Mammary	41
	Skin	41
ENA-78	Epithelial cells (lung)	45
IP10	Monocytes	48, 49, 147
	Endothelial cells (HUVEC)	48, 49, 169
	Fibroblasts	
	Lung	391
	Skin	48, 49
	Keratinocytes	49
	Synovium (fibroblasts)	391
	Tumor cells (melanocytes)	436
MCP-1	Monocytes	55, 58, 61, 65, 75, 148–152, 171
	Endothelial cells	
	Aorta	170
	HUVEC	60, 166, 168, 169, 171
	Lung	150
	Epithelial cells	
	Retina	177
	Lung	150, 176
	Fibroblasts	
	Lung	201, 391, 437
	Skin	168, 391, 437
	Chondrocytes	185

(*continued*)

TABLE II *Continued*

Chemokine[a]	Source	Ref.
	Keratinocytes	107, 438
	Mesangial cells	430, 439, 440
	Osteoblasts	70
	Smooth muscle	
	Lung	150
	Vascular	68, 170, 433
	Synovium	
	Fibroblasts	391, 395
	Macrophages	395
	Synoviocytes	184, 441
	Tumor cells	
	Fibrosarcoma	187
	Glioma	56, 68
	Histiocytoma	442
	Melanoma	189, 193
	Osteosarcoma	66, 68, 75
	Sarcoma	188
HC14	Monocytes	65
	Tumor cells (osteosarcoma)	66
MCP-3	Monocytes	67
	Tumor cells (osteosarcoma)	66
MIP-1α	U937, HL-60	93
	T lymphocytes	51, 154, 155, 443
	B lymphocytes	155
	Fibroblasts	
	Oral cavity	93
	Synovium	391
	Tumor cells	
	Hematopoietic	443
	Glioma	93
MIP-1β	Monocytes	52, 133, 153
	T lymphocytes	52, 62, 153–155
	B lymphocytes	52, 155
	Fibroblasts (lung)	391
	Synovium (fibroblasts)	391
I-309	T lymphocytes	62, 63
RANTES	T lymphocytes	62, 64, 154
	Platelets	253

[a] Synonyms and isoforms: IL-8—MCNDF, NAF, NAP, NAP-1, CGP; GROα—MGSA; MCP-1—GDCF, HC11, LDCF, MCAF, TSG-8; HC14—MCP-2; MIP-1α—AT464, GOS-19, LD78; MIP-1β—Act-2, AT744, G-26, H400, HC21, MAD-5.

regulin up-regulates the IL-8 gene by enhancing the binding of NF-κB in the IL-8 promoter region (181). It is possible that the effects of some of the stimuli are mediated via the expression of IL-1 or TNF.

Chemokine production has been intensively studied in *keratinocytes* as well as in *synovial* and *mesangial cells*, where IL-1 and TNF induce the production of IL-8, GROα, and MCP-1 (Table II). Of particular interest are the reports by Villiger *et al.* describing the expression of MCP-1 in synoviocytes after stimulation with IL-1β, TNFα, LPS, platelet-derived growth factor, or transforming growth factor-β (184), and in chondrocytes, which respond, in addition, to leukemia inhibitory factor (185). In these studies the MCP-1 isoforms of 13 and 15 kDa and a minor isoform of 15.2 kDa were identified in the cell supernatants (184, 185).

CXC and CC chemokines are often constitutively expressed in transformed cells, and the medium of such cultures has frequently been used for the isolation and characterization of different chemokines (36, 56, 68, 75, 186–189). The regulation of expression has not been thoroughly studied, although transformed cells respond to stimulation (190–193). MCP-1 may be involved in inhibition of tumor growth (194–197), whereas GROα has been proposed as an autocrine growth stimulatory factor (37).

C. Regulation

Modulation of the expression of chemokine genes has been studied mainly in monocytes and tissue cells. IL-8 production in blood monocytes is inhibited by dexamethasone (127, 198), IFN-γ (127), IL-4 (103), and IL-10 (199). 1,25-Dihydroxycholecalciferol inhibits IL-8 expression in monocytes, keratinocytes, and fibroblasts (99) (see Section III,B). Dexamethasone is probably one of the most potent inhibitors of CXC and CC chemokine expression in that it also affects fibroblasts (200, 201), synoviocytes (184), chondrocytes (185), and mesangial cells (202). The action on fibroblasts has been shown to depend on mRNA and protein synthesis (200). Dexamethasone, on the other hand, does not affect IL-8 production in monocytes stimulated with PMA (198) and fibroblasts stimulated with leukoregulin (181). Leukoregulin-dependent IL-8 expression in fibroblasts is also insensitive to retinoic acid or transforming growth factor-β1 (181). Indomethacin and other nonsteroidal anti-inflammatory drugs are ineffective (127), whereas prostaglandin E_2 (PGE_2) has been reported to inhibit MCP-1 expression in lung fibroblasts (201). MIP-1β gene expression induced in human monocytes by IL-7 and LPS is also insensitive to dexamethasone, but is inhibited by IL-4 (153). Retinoic acid induces

IL-8 in skin fibroblasts and acts synergistically with PMA (203). In synovial cells and chondrocytes, MCP-1 production is observed following activation of protein kinase A by cAMP and protein kinase C by PMA, and is enhanced by retinoic acid, which also potentiates the effects of IL-1 and LPS (184, 185).

The effects of interferons are potentially interesting. It has been pointed out that IFN-γ induces IP10, but tends to inhibit the expression of all other CXC chemokines, IL-8 in particular, as shown, for instance, in thymic epithelial cells (104). In keratinocytes and monocytes, however, IFN-γ moderately stimulates MCP-1 production and synergizes with TNFα (107, 148). IFN-γ may also have indirect effects, as shown in a study in which IFN-γ-dependent up-regulation of MHC class II molecules rendered fibroblast-like synoviocytes capable of producing IL-8 in response to challenge with the superantigen staphylococcal enterotoxin A (204). Another remarkable observation was reported by Oliveira *et al.* (180), who showed that IFN-β inhibits the transcriptional activation of the IL-8 gene in fibroblasts stimulated with TNF without affecting the expression of other TNF-inducible genes.

Cycloheximide has been used to study the mechanism of CXC and CC chemokine induction in various cells. A superinduction by cycloheximide is observed for expression of IL-8 in monocytes (148), endothelial cells (166), fibroblasts (181), and synoviocytes (204), as well as for MCP-1 expression in endothelial cells (148, 166), smooth muscle cells, and fibrosarcoma cells (148). In IL-1-, TNF-, or LPS-stimulated monocytes, MCP-1 production is inhibited by cycloheximide (148).

V. Biological Activities

A. Neutrophils

1. Interleukin-8

IL-8 was identified as an agonist for neutrophils on the basis of two *in vitro* effects, chemotaxis and the release of granule enzymes (15, 16, 120). Subsequently, the biological activities of IL-8 on neutrophils and the mechanism of signal transduction were studied in great detail and compared with the effects of well-characterized neutrophil attractants such as C5a, fMet-Leu-Phe, PAF, and LTB_4. Shape change (which is a correlate of chemotaxis), the transient rise of the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), exocytosis, and respiratory burst were assessed in real time using sensitive methods (205–207). In addition, the

up-regulation of adhesion proteins and the formation of bioactive lipids were investigated. The comparison shows that IL-8 behaves like a classical chemotactic agonist.

a. Shape Change

The shape change reflects activation of the contractile cytoskeleton and is related to neutrophil motion (208). It is monitored and quantified in cell suspensions by laser nephelometry. Adding a chemoattractant induces a transient increase in light transmission and a concomitant decrease in right-angle light scattering. The cells protrude large, thin cytoplasmic lamellae, and their body volume decreases slightly (209). The optical transient is rapid; the peak is reached in 20 to 30 seconds and control levels are restored within a few minutes. The neutrophil shape change responses to IL-8 and C5a are practically identical in extent and last for 1.5 to 2 minutes, whereas the effect of fMet-Leu-Phe is more prolonged (210). Like other chemoattractants, IL-8 also induces a rapid polymerization of actin (211). When the neutrophils are prestimulated (and presumably synchronized) with low concentrations of phorbol myristate acetate or a chemotactic agonist, the shape-related optical response to IL-8 or other chemoattractants shows regular oscillations with a period of about 8 seconds, which presumably reflects the periodic protrusion and retraction of lamellipodia (209). As shown for fMet-Leu-Phe, the optical signal oscillates in parallel with the cellular content of filamentous actin, suggesting that the oscillations are related to cyclic assembly and disassembly of contractile filaments (212, 213).

b. Exocytosis

IL-8 induces the release of enzymes and other proteins from intracellular storage organelles. Exocytosis from specific granules and secretory vesicles, as assessed by measuring vitamin B_{12}-binding protein and gelatinase (73, 214), is observed in normal, freshly isolated neutrophils. On pretreatment of the cells with cytochalasin B, the release of elastase, β-glucuronidase, myeloperoxidase, and other enzymes from the azurophilic granules is also observed (15, 73).

c. Receptor Up-Regulation and Adhesion

The release of soluble granule components is accompanied by plasma membrane changes through fusion with the storage organelle membranes. During IL-8-dependent exocytosis of specific granules, two integrins, complement receptor type 3 (CR3, CD11b/CD18) and p150,95 (CD11c/CD18) are up-regulated (215, 216). IL-8 also causes

an increased surface expression of complement receptor type 1 (CR1) by mobilization from a secretory compartment distinct from specific granules (215, 217). Up-regulation by IL-8 increases the capacity of CR3 to bind several ligands, including complement component C3bi-tagged substrates. CR3 recognizes C3bi, fibrinogen, and a still unidentified endothelial ligand. IL-8 promotes the adherence of neutrophils to fibrinogen-coated surfaces and monolayers of human umbilical vein endothelial cells (216, 218). Through a different binding domain CR3 also recognizes lipid A. IL-8 enhances neutrophil binding to lipid A-coated erythrocytes, and is, therefore, likely to increase the interaction between neutrophils and bacteria (216).

Gimbrone and co-workers have reported that IL-8 inhibits the adhesion of neutrophils to LPS-activated human umbilical vein endothelial cell monolayers (24). The inhibition is mediated by a direct effect of IL-8 on the neutrophils, and is independent of the adhesive properties of the endothelium (219). Since other chemoattractants have similar effects, it has been suggested that the inhibition is related to the shedding of L-selectin (220). Other mechanisms, however, cannot be excluded since IL-8 also induces the detachment of neutrophils that have adhered to an endothelial monolayer, and presumably have already shed L-selectin (211).

d. Formation of Bioactive Lipids

IL-8 activates neutrophil 5-lipoxygenase as shown by the formation of LTB_4, its omega-oxidized metabolites (20-OH- and 20-COOH-LTB_4), and 5-hydroxy-eicosatetraenoic acid (5-HETE) from exogenous arachidonate (221). Under similar conditions, acetyl-CoA:1-alkyl-2-lyso-*sn*-glycero-3-phosphocholine acetyltransferase is also activated, and a rapid, concentration-dependent synthesis of PAF is observed. Newly produced PAF is partly released and partly remains associated with the neutrophils (222).

e. Respiratory Burst

The respiratory burst reflects the activation of NADPH-oxidase, a membrane-bound electron transport chain that oxidizes cytosolic NADPH and reduces extracellular oxygen to superoxide (205). As shown by real-time recordings of H_2O_2 (the product of superoxide dismutation), the onset of the burst is independent of the chemotactic agonist used, whereas the duration of the response varies greatly (223). The burst responses to PAF and LTB_4 are terminated much more rapidly than those elicited by fMet-Leu-Phe or C5a, and the total yield of H_2O_2 is 30- to 50-fold lower. There has been some controversy about

the ability of IL-8 to induce the respiratory burst (224). Using the appropriate methodology, however, a burst of O_2^- or H_2O_2 production is regularly detected when preparations of human neutrophils that respond to fMet-Leu-Phe are stimulated with IL-8 (225). Neutrophil responsiveness to IL-8 can be markedly enhanced by pretreatment with concanavalin A (226).

2. *Other CXC Proteins*

Most CXC chemokines activate neutrophils. NAP-2 (34), GROα (38, 39), GROβ, GROγ (44), and ENA-78 (45) have been compared with IL-8 in our laboratory. They all have the characteristic properties of chemotactic receptor agonists and induce cytosolic free calcium changes, shape changes, chemotaxis, and exocytosis in the same molar range as IL-8, with threshold concentrations of 0.3 to 1 n*M*. The extent of granule exocytosis, however, is only about 50% of that observed with IL-8, and the respiratory burst response, although significant, is very weak (34, 38, 44, 45). Pretreatment with agents that prime the neutrophils markedly enhances responsiveness and can be used to show that all CXC chemokines are able to activate the NADPH-oxidase (226). TNF, a well-known priming agent, was reported to enhance neutrophil exocytosis after stimulation with IL-8 and NAP-2 (227).

PBP, CTAP-III, and PF4, the CXC proteins that are stored in platelet α-granules and are released in bulk on platelet activation, are virtually inactive toward neutrophils (34, 35). As already indicated, CTAP-III is converted into a neutrophil-activating derivative, NAP-2, by N-terminal processing. PF4 lacks neutrophil-stimulating properties comparable to those of IL-8 and NAP-2 (34, 228). Chemotaxis and exocytosis described in former reports were obtained at PF4 concentrations that were at least 1000-fold higher than those required for IL-8 (229–231). PF4 has been studied extensively in the context of thrombosis and circulatory disorders (232). Its affinity for heparin and other sulfated glycans suggests that it may function as a prothrombotic agent. Effects of PF4 on cellular functions, such as inhibition of megakaryocytopoiesis (233) and angiogenesis (234), as well as modulation of cellular immune responses (232), have been described, but as yet no cell surface receptor has been identified. The finding that PF4 modulates growth factor receptor interactions (235, 236) suggests that at least some of its effects are indirect.

MIP-1α (but not MIP-1β) induces a moderate transient rise in $[Ca^{2+}]_i$ and shape change in neutrophils, but induces no functional responses such as chemotaxis or exocytosis (237, 238). This observation appears worth persuing because MIP-1α is so far the only CC

chemokine with at least some stimulatory effect on neutrophils. In this context, it must be remembered that Wolpe *et al.* (53) reported that murine MIP-1α stimulates human neutrophils.

B. Basophils

1. CXC Chemokines

IL-8 induces the release of histamine (239, 240) and peptidoleukotrienes (240) from human blood basophils. Release is largely dependent on the pretreatment of the cells with IL-3, IL-5, or GM-CSF (241). The IL-8 effects on basophils are observed at concentrations that also induce exocytosis of azurophilic and specific granules from cytochalasin B-treated neutrophils (240). The responses are rapid: half-maximal release occurs within 1 minute and the maximum is reached in 5 to 10 minutes. When the cells are not pretreated with IL-3 or GM-CSF, only low levels of histamine, but no leukotrienes are released after stimulation with IL-8 (240). It was shown that human basophils bear IL-8 receptors that signal through *B. pertussis* toxin-sensitive G-proteins and weakly bind NAP-2, but not CTAP-III or PF4 (242). IL-8 and, at high concentrations, NAP-2 induce a rapid, transient $[Ca^{2+}]_i$ rise in neutrophil-free preparations of basophils, whereas CTAP-III and PF4 are inactive (242). These observations are in disagreement with a study showing that both NAP-2 and CTAP-III induce histamine release from human basophils (243). It must be kept in mind, however, that CXC proteins are cationic and, at high concentrations, may induce exocytosis due to their charge, as shown by the release obtained with poly(D-lysine), histones, and lysozyme, which is even superior to that induced by CTAP-III and PF4 (242). IL-8, GROα, GROβ, and GROγ are also chemotactic for basophils (44).

Kuna *et al.* (244) reported that IL-8, at low concentrations, inhibits histamine release induced in basophils by a "histamine-releasing factor" (HRF) preparation, CTAP-III or IL-3. In an analogous study, we found that IL-8 *inhibits* the release of histamine and leukotrienes by basophils pretreated with IL-3 at concentrations that are 10- to 100-fold lower than those required for the *induction* of release. This effect appears to be stimulus restricted since the responses to C5a, fMet-Leu-Phe, or anti-IgE are not modified (245).

2. CC Chemokines

CC chemokines are much more powerful as stimuli of basophils than are CXC chemokines. Three independent studies reported that MCP-1 induces a rapid and marked release of histamine from human basophils at concentrations ranging between 1 and 100 n*M* (246–248). MCP-1 is

highly effective on normal cells, and its activity is even more pronounced after pretreatment with IL-3, IL-5, or GM-CSF (246, 248). As reported by Bischoff *et al.* (248), MCP-1 also induces the release of peptidoleukotrienes, provided that the basophils are pretreated with IL-3, IL-5, or GM-CSF. MCP-1 is considerably more potent and more effective than IL-8 or C3a, but inferior to C5a. Stimulation of basophils appears to depend on G-protein-coupled receptors, as suggested by the transient rise in $[Ca^{2+}]_i$ and the sensitivity to *B. pertussis* toxin (248). No cross-desensitization is observed between MCP-1 and IL-8, indicating that they act by different receptors (248). Remarkable effects are also obtained with two other CC chemokines, RANTES (241, 249) and MIP-1α (241, 250), which stimulate murine mast cells as well (250), whereas MIP-1β is apparently inactive on basophils (241). Direct comparison shows that MCP-1 is more effective as inducer of histamine and leukotriene release, whereas RANTES is most effective as chemoattractant (241).

C. Eosinophils

Much less information is available about the action of chemokines on eosinophils. In a study on the activation of eosinophils by chemotactic agonists, using cells from patients with hypereosinophilic syndrome, Kernen *et al.* (251) showed that IL-8 induces $[Ca^{2+}]_i$ transients, shape change, and the release of eosinophil peroxidase. The activity of IL-8, however, is markedly lower than that of C5a and PAF. It was also reported that IL-8 induces eosinophil chemotaxis *in vitro* when the cells are pretreated with IL-3 or GM-CSF (252).

Like basophils, eosinophils are more responsive to CC than to CXC chemokines. RANTES is a powerful chemoattractant with maximum activities between 10 and 30 n*M* (253, 254). Its efficacy, as judged by the percentage of migrating cells, is similar to that of C5a, and is two- to threefold higher than that of MIP-1α (254). RANTES also induces exocytosis of eosinophil cationic protein from cytochalasin B-treated cells and the respiratory burst, whereas MIP-1α induces exocytosis only (254). MCP-1 (a potent stimulus of basophils and monocytes) and MIP-1β are inactive on eosinophils. As indicated by desensitization of the $[Ca^{2+}]_i$ changes induced by repeated stimulation, MIP-1α and RANTES partially share receptors on eosinophils (254).

D. Monocytes

Several studies convincingly show that IL-8 is not chemotactic for monocytes (16, 120, 255). Monocytes, however, saturably bind IL-8 labeled with fluorescein (256) or radioactive iodine (257), albeit to a lesser extent than neutrophils. In addition, sensitive real-time mea-

surements demonstrate that IL-8 elicits minor but significant $[Ca^{2+}]_i$ changes and the respiratory burst in human blood monocytes (226). Similar but even weaker responses are obtained with GROα. Pretreatment of the monocytes with concanavalin A enhances the respiratory burst response to IL-8 and GROα more than 10-fold (226). In an early study, PF4 was reported to be chemotactic for human neutrophils and monocytes *in vitro* (229), but this observation has not been confirmed.

Monocytes, by contrast, are highly responsive to CC chemokines. MCP-1 (55, 56, 258, 259), RANTES (260), I-309 (261), HC14 (also called MCP-2), and the newly described chemokine MCP-3 (66) attract monocytes *in vitro*, and RANTES, MCP-1, and MIP-1α attract the human monocytic leukemia cells THP-1 (262). MCP-1, HC14 (MCP-2), and MCP-3 also induce the selective infiltration of monocytes on intradermal injection in rats (187) and rabbits (66). $[Ca^{2+}]_i$ changes are observed in monocytes stimulated with MCP-1 (241, 258, 259), I-309 (261), RANTES (241), MIP-1α (238, 241), and MIP-1β (238). In monocytes MCP-1 also induces the respiratory burst (187, 258), expression of the β_2-integrins CD11b/CD18 and CD11c/CD18 (but not CD11a/CD18), and the production of IL-1 and IL-6 (263).

Matsushima *et al.* (57) observed that growth of several tumor cell lines cultured in the presence of human blood monocytes is inhibited by the addition of MCP-1. Growth retardation was observed after 2 to 3 days, and tumor cell lysis after 5 days (57). MCP-1 generated by tumor cells (56, 68, 189, 194, 264) could be involved in the recruitment of macrophage-dependent antitumor defense. Experiments with transplanted sarcoma clones in mice show that the number of tumor-associated macrophages is related to MCP-1 production by the tumor, and that clones expressing high levels of MCP-1 grow more slowly (195). Similar observations were made in mice receiving murine melanoma cells expressing MCP-1 through gene transfer (196). Another study shows that Chinese hamster ovary (CHO) cells engineered to express MCP-1 lose their ability to form tumors in nude mice and inhibit the growth of coinjected, nonengineered HeLa or CHO cells (197).

E. Lymphocytes

The term IL-8 was originally proposed in a paper reporting that this neutrophil-activating protein was chemotactic for human T lymphocytes *in vitro*, and elicited lymphocyte infiltration on local injection in rats (265). Subsequent studies reported *in vitro* chemotactic activity of IL-8 for human lymphocytes with a presumed preference for T cells (256, 266). No $[Ca^{2+}]_i$ changes, however, are observed in blood lym-

phocytes exposed to IL-8 at maximum effective concentrations for neutrophils (210, 267). Bacon *et al.* (267) report a rapid and long-lasting increase in inositol phosphates in lymphocytes after stimulation with IL-8 or phytohemagglutinin, which (in contrast to IL-8) also enhances $[Ca^{2+}]_i$. It has been suggested that IL-8 receptors occur only on subsets of T lymphocytes (265), but the existence of such receptors remains questionable (257, 268). Low numbers of transcripts for IL-8R1 (the IL-8-selective receptor; see Section VI,B,1) are detectable by reverse transcriptase polymerase chain reaction (RT-PCR) in $CD4^+$ T cells, blood lymphocytes treated with PHA, and Jurkat cells (269). According to Wilkinson and Newman (270), human lymphocytes respond to IL-8 only after activation, e.g., by exposure to an antibody against CD3 (anti-CD3) or to a purified protein derivative of *Mycobacterium tuberculosis*. IL-8 was also reported to induce locomotion of natural killer cells after activation with IL-2 (271). In contrast to former evidence (265, 266, 270), a recent comparison of CXC and CC chemokines shows that IL-8 is not chemotactic for T cells, either unfractionated or after separation according to the $CD4^+$ and $CD8^+$ phenotype (272). This finding is in agreement with the observation that IL-8 does not attract lymphocytes on intradermal injection in humans (273, 274).

It was shown a few years ago that RANTES is chemotactic for T lymphocytes of the memory type, but not for neutrophils (260). Other CC chemokines were studied recently by Taub *et al.* (272), who confirm the action of RANTES and show that human T lymphocytes respond to MIP-1α and MIP-1β as well. RANTES acts on resting and activated T cells, whereas MIP-1α and MIP-1β are effective on anti-CD3-stimulated cells only. Much weaker migration is observed with IL-2, but MCP-1, PF4, and IL-8 are inactive (272). MIP-1α is more effective toward $CD8^+$ (suppressor and cytotoxic) T lymphocytes and MIP-1β toward $CD4^+$ (helper) T lymphocytes, whereas RANTES attracts both (272). These results, however, are in disagreement with observations by Tanaka *et al.* (275) that MIP-1β, but not MIP-1α, is chemotactic for resting T cells and enhances the adherence of $CD8^+$, but not $CD4^+$, cells to VCAM-1. In contrast to RANTES or GROα, which are inactive, MIP-1β promotes T cell adhesion to VCAM-1 when bound to heparin or proteoglycans, suggesting that it may exert its effects while immobilized on the surface of endothelial cells (275).

A totally different activity on lymphocytes was described by Kimata *et al.* (276), who showed that IL-8 inhibits the IL-4-stimulated production of IgE by B lymphocytes in an apparently selective manner, without affecting the production of other immunoglobulins.

F. Other Cells

A major effort has been made to study potential functions of CXC and CC chemokines on cells other than leukocytes. Particular attention has been devoted to cell growth because one of the CXC chemokines, GROα, was originally reported as a product of a growth-regulated gene (82). GROα was purified from the medium of cultured melanoma cells and was termed "melanoma growth-stimulatory activity" because of mitogenic effects on normal and transformed human melanocyte cell lines (36, 277, 278) (see Section II,A). Cultures established from biopsies of primary melanomas and metastases constitutively produce GROα and several other cytokines, including IL-8 and MCP-1 (36, 189, 279), which may act in auto- or paracrine fashion. GROα high-affinity binding sites, which appear to be different from chemokine receptors on leukocytes, are present in large number on the human melanoma cell line Hs294T (see Section VI,A,1) (280). Melanoma cells have been reported to migrate *in vitro* in response to IL-8 by a mechanism that was characterized as haptotaxis (281).

Other functions of chemokines related to growth and tissue remodeling have been described, and may be of interest in inflammation. GROα and IL-8, but none of the CC chemokines, were shown to inhibit collagen expression in synovial fibroblasts from patients with rheumatoid arthritis (282), suggesting that CXC chemokines, possibly in concert with tissue cell growth factors, may participate in tissue remodeling and repair. Enhanced expression of the GROα homologues is observed in rats (42) and chickens (283) in wound tissue, tumors, and the surroundings of implants, and may reflect repair activities and neovascularization. Human IL-8 was reported to be angiogenic in the rat cornea and to induce the migration of human umbilical vein endothelial cells *in vitro* (284). Chemotaxis of epidermal cells in response to IL-8 has also been observed (285). Interestingly, PF4 inhibits angiogenesis and endothelial cell proliferation (234) and was reported to retard the growth of melanoma and colon carcinoma in mice (286).

Murine MIP-1α and MIP-1β enhance the proliferation of committed murine granulocyte–macrophage progenitor cells in the presence of GM-CSF or CSF-1 (287). The proliferation of less mature progenitor cells that depend on IL-3, by contrast, is suppressed by MIP-1α (288), which was also described as a stem cell inhibitor (289). This effect of MIP-1α is prevented by MIP-1β (290), illustrating the potential complexity of chemokine functions in hematopoiesis (291).

G. Effects *in Vivo*

The demonstration of leukocyte recruitment *in vivo* was essential for the assessment of the potential role of IL-8 and other chemokines in pathology. Intradermal injection of IL-8 in rabbits induces plasma exudation and a massive and exclusive local infiltration of neutrophil leukocytes (292–294). Histology shows that neutrophil accumulation is particularly prominent within and around venules of the upper and lower dermis. Plasma exudation depends on the recruitment of neutrophils, and is not observed in neutrophil-depleted animals (294, 295). The effectiveness of IL-8 is strongly enhanced by vasodilating agents. In the presence of PGE_2, significant neutrophil infiltration is observed on injection of as little as 10^{-14} to 10^{-13} mol IL-8 per site; in its absence the threshold level is about 10^{-11} mol per site (292, 293). PGE_2 also markedly increases the number of accumulating neutrophils and the volume of extravasating plasma (296).

The action of IL-8 is direct, i.e., not mediated by the local *de novo* induction of a chemoattractant, because no changes are observed on coinjection of actinomycin D (292). The intradermal effect of IL-8 is rapid and long lasting. The maximum rate of neutrophil influx is reached within 30 minutes and migration continues for at least 8 hours (295). This finding indicates that IL-8 is resistant to inactivation and is retained at the site of injection, in contrast to chemoattractants such as fMet-Leu-Phe, C5a, or PAF, which are rapidly metabolized. As a cationic protein IL-8 binds to glycosaminoglycans of the tissue matrix and cell membranes, and is thus likely to persist in active form in the microenvironment of the cells from which it is released. We have recently shown that association with heparan sulfate enhances the chemotactic activity of IL-8 *in vitro* (297, 298) suggesting that CXC chemokines can act in substrate-bound form. Neutrophil accumulation *in vivo* is also obtained with NAP-2 (299) and GROα (38).

Intradermal injection of IL-8 in humans induces a time-dependent perivascular neutrophil infiltration that lasts for several hours (273, 274). Most migrated cells are morphologically intact with little evidence of degranulation. No basophils and eosinophils are found, and the number of lymphocytes is not increased over control values, as shown by immunohistology. Interestingly, the injection of IL-8 causes no wheal and flare, itching, or pain, suggesting that it does not elicit histamine release from local mast cells, as observed after injection of C5a (274). The selectivity of IL-8 is further supported by a study of Colditz and Watson (300), who quantified the influx of radiolabeled neutrophils and lymphocytes into skin sites of sheep after injection of

different attractants, and found neutrophil-to-lymphocyte ratios of 45 for IL-8 and C5a, 5 for TNFα and IL-1α, and 0.1 for IFN-γ.

Infiltration of monocytes is observed in skin sites treated with MCP-1 (66, 187). Similar degrees of monocyte recruitment were obtained after intradermal injection of 10 to 500 ng per site of MCP-1, MCP-2 (HC14), or MCP-3 in rabbits (66). The infiltrating cells are almost exclusively monocytes and have a tendency to form clusters. Neutrophils are seen only occasionally.

High plasma levels of IL-8 are observed in septic shock or on systemic administration of LPS or IL-1. After injection of a lethal dose of *E. coli* or LPS, a rise of TNFα and in some instances IL-1β precedes the longer lasting increase of IL-8 and IL-6 (301). Intravenous administration of IL-8 either as a bolus or by infusion for 8 hours in baboons leads to a rapid, transient neutropenia (302). Neutrophil counts drop to 1–10% of control within 1 minute. Recovery after 10–15 minutes is followed by granulocytosis of nearly 200% which lasts for the whole duration of the IL-8 infusion and normalizes thereafter within a few hours. IL-8 does not induce the formation of TNF, IL-1, or IL-6, and has no appreciable hemodynamic effects even when the plasma concentration is two to three times higher than in septic shock. Apart from some neutrophil margination in the lung, histology of several tissues, including the bone marrow, is normal. The number of band cells does not increase, thus it is suggested that IL-8-dependent neutrophilia is due to demargination rather than to increased release from the bone marrow (302).

VI. Receptors

A. Biochemical Studies

1. Receptors for CXC Chemokines

Receptors for IL-8 were first identified by binding studies performed with human neutrophils. Several independent groups reported the existence of a single class of high-affinity receptors with K_d values between 0.2 and 4 n*M*, and densities of 20,000 to 90,000 sites per cell (257, 268, 303, 304) (Table III). Receptors with a 10-fold higher affinity (5000 sites per cell, K_d = 11–35 p*M*) were also described, but this observation has not been confirmed (257, 304). At 37°C, bound ^{125}I-labeled IL-8 is rapidly internalized and degraded (257, 268, 305). More than 90% of the ligand-bound receptors are endocytosed within 10 minutes and the receptors are recycled as indicated by their reexpression on the cell surface even in the presence of cycloheximide (305).

TABLE III
CELLULAR EXPRESSION OF RECEPTORS FOR CHEMOKINES[a]

Cell	IL-8 [1]	GROα[b] [2]	NAP-2[b] [2]	MCP-1 [3]	MIP-1α [4]	MIP-1β [5]	RANTES [6]
Neutrophils	$2–9 \times 10^4$, (T)	$2–3 \times 10^4$	$2–3 \times 10^4$				
HL-60	(T)	(T)	(T)		(T)	+	
HL-60-d[c]	8×10^3, (T)	(T)	(T)		(T)	+	
K562	(T)					1×10^4	
Monocytes	$2–8 \times 10^3$, (T)	(T)	(T)	$1–5 \times 10^3$	+		570
U937	$3–5 \times 10^3$, (T)				(T)		
Monomac 6	4×10^3						
THP-1	2×10^3			4×10^3	660, (T)		$0.7–5 \times 10^3$
AML 193	(T)	(T)	(T)				
Macrophages				4×10^3			
Basophils	$4–10 \times 10^3$						
PBLs	2×10^3					+	
PBLs (PHA/PMA)						5×10^4	
T lymphocytes	(T)						
Jurkat	(T)						
MT-2						1×10^4	
B lymphocytes					(T)		
Erythrocytes	2×10^3	+	+	2×10^3			+
Placenta		+					
Melanocytes	(T)	(T)	(T)				
Melanomas	(T)						
Hs294T		5×10^4					

[a] References to the receptors for the chemokines are [1], 39, 242, 257, 268, 269, 303–305, 322, 323, 335; [2], 39, 44, 269, 280, 304, 306, 307, 309, 316, 335; [3], 187, 310–312, 316; [4], 237, 262, 314; [5], 313; [6], 262, 312, 314.

[b] For GROα and NAP-2 only high-affinity binding sites are listed. The numerical values given in the columns refer to binding sites per cell. The symbol (T) indicates expression of transcripts, based on Northern analysis or RT-PCR, for the cloned receptors (IL-8R1, IL-8R2, or MIP-1αR). Plus signs indicate the presence of binding sites from studies in which the binding parameters were not determined.

[c] HL-60 differentiated toward neutrophils by treatment with DMSO, retinoic acid, or dibutyryl cAMP.

IL-8 receptors are selective for CXC chemokines that act on neutrophils (see later). They do not bind unrelated chemoattractants such as fMet-Leu-Phe, C5a, LTB_4, and PAF (268, 304), or the nonchemotactic CXC proteins PF4 (304) and IP10 (47). Competition studies show that radiolabeled IL-8 is displaced by NAP-2 and GROα (304) (Fig. 3). Analysis of the data reveals the existence of two IL-8 receptors: one with high affinity for all three ligands (K_d = 0.1–0.4 n*M*) and one with high affinity for IL-8 and low affinity for NAP-2 and GROα (K_d > 100 n*M*) (304). High- and low-affinity receptors for NAP-2 were also described by Schnitzel *et al.* (306). These observations are confirmed by direct binding studies using synthetic GROα and NAP-2 with C-terminal tyrosine substitutions introduced for iodination (307). These

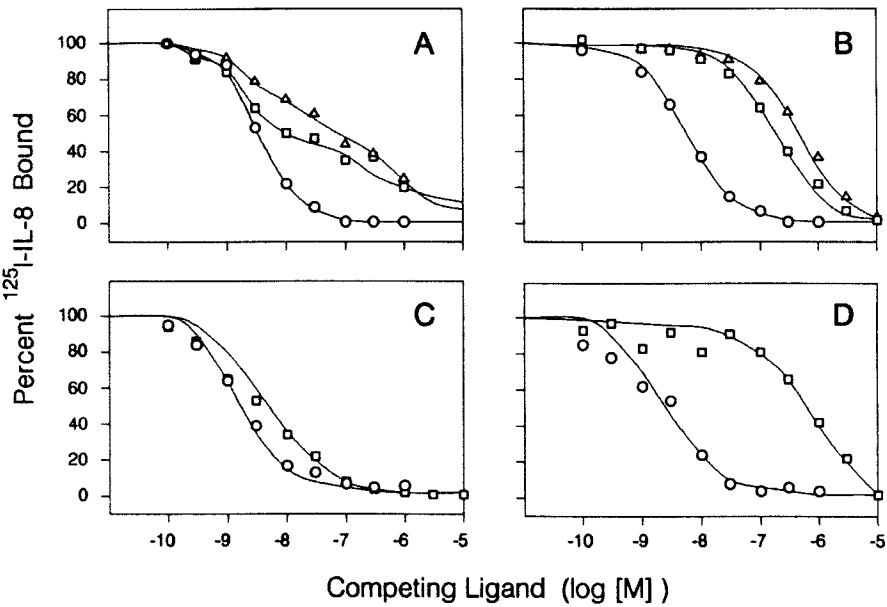


FIG. 3. Binding competition analysis of the natural and recombinant IL-8 receptors, IL-8R1 and IL-8R2. Binding of ^{125}I-labeled IL-8 to human neutrophils (A), solubilized neutrophil membranes (B), and E293 cells expressing IL-8R2 (C) or IL-8R1 (D) incubated on ice in the presence of increasing concentrations of unlabeled IL-8 (○), GROα (□), or NAP-2 (△). Binding of 100% refers to the specific ^{125}I-labeled IL-8 binding in the absence of unlabeled competing ligands. (See Refs. 304, 307, and 335 for experimental information.)

experiments show that 30–45% of the receptors bind these ligands with high affinity (K_d = 0.3–0.7 n*M*) and 55–70% with an approximately 100-fold lower affinity. Additional binding sites with single specificities, e.g., selective receptors for GROα or NAP-2, are not found on neutrophils. Two neutrophil membrane proteins (44–59 and 67–70 kDa) that bind IL-8, NAP-2, and GROα can be demonstrated by cross-linking with iodinated IL-8 (303, 304), GROα, and NAP-2 (307). Competition with radiolabeled IL-8, GROα, or NAP-2 shows that both proteins recognize all three CXC chemokines (304, 307). Solubilization of neutrophil membranes with digitonin apparently inactivates the 67- to 70-kDa protein, and competition experiments show that the 44- to 59-kDa protein can be solubilized in active form and corresponds to the receptor with high affinity for IL-8 and low affinity for GROα and NAP-2 (307). A single neutrophil protein of 58 kDa that cross-links IL-8 was described by Grob *et al.* (268).

The sharing of IL-8 receptors by CXC chemokines is clearly demonstrated by desensitization experiments. As shown by $[Ca^{2+}]_i$ recordings, stimulation of neutrophils with IL-8 results in a marked attenuation of the response to the subsequent challenge with NAP-2 or GROα. A similar degree of cross-desensitization is observed on sequential stimulation with NAP-2 and GROα or vice versa (226, 304). Stimulation with NAP-2 or GROα also attenuates the response to IL-8, although in this case desensitization is only moderate, presumably because IL-8 can still freely interact with receptors that bind NAP-2 and GROα with low affinity (304). Studies show that GROβ, GROγ (44), and ENA-78 (45) also share receptors with IL-8, and behave like GROα and NAP-2 in desensitization experiments.

A first indication that IL-8 receptors are coupled to GTP-binding proteins (G-proteins) came from the observation that pretreatment with *B. pertussis* toxin (which inactivates G_i-type G-proteins by ADP-ribosylation) renders the neutrophils unresponsive to IL-8 (210). It was later shown that IL-8 induces GTP hydrolysis in neutrophil membranes by stimulation of the intrinsic GTPase activity of the G-protein α-subunit (308). In addition, IL-8 stimulates high-affinity binding of GTPγ[^{35}S] to the membranes, indicating that GDP is exchanged for GTP. GROα and NAP-2 are less effective, as would be expected from the binding experiments (308). Coupling of the G-protein to the receptor is essential for high-affinity binding of IL-8. Treatment of neutrophil membranes with the nonhydrolyzable analog, GTPγS, prevents the coupling and leads to a marked drop in binding affinity for IL-8, GROα, and NAP-2 (307).

Binding and competition studies with radiolabeled ligand reveal a single class of high affinity IL-8 receptors (3500 to 9600 per cell; K_d = 0.15 n*M*) on human blood basophils, which also weakly bind NAP-2 (242). Two proteins of 58 and 78 kDa that bind ^{125}I-labeled GROα were described in human placental membrane preparations (309), but the binding of IL-8 or other CXC chemokines was not tested. A receptor for GROα (K_d = 4 n*M*; > 50,000 sites per cell) that does not bind IL-8 was recently reported in the human melanoma cell line, Hs294T (280). Further studies are required to establish whether the melanocyte growth-stimulatory activity of GROα reported in Hs294T cells by Richmond *et al.* (37) is mediated through this receptor.

2. *Receptors for CC Chemokines*

Much less information is available on CC chemokine receptors (Table III). Yoshimura and Leonard (310) described MCP-1 receptors on human monocytes (K_d = 2 n*M*; 1100 to 2300 sites per cell), and similar findings were reported subsequently for monocytes and THP-1 cells

(K_d values between 0.5 and 1.1 n*M*; 1600 to 4000 sites per cell) (311, 312). Using a MCP-1 derivative with three tyrosine residues added at the C-terminus for iodination, we found on average about 5000 receptors on human monocytes and 3600 receptors on monocyte-derived macrophages (K_d = 9 n*M*), but no binding to neutrophils, blood lymphocytes, U937, and HL-60 cells. Treatment of monocyte membranes with GTPγS results in a more than 20-fold decrease in affinity, suggesting that high-affinity binding requires G-protein coupling.

Receptors for MIP-1β (Act-2) were identified on activated human blood lymphocytes (313). PHA/PMA stimulation of resting cells, which possess only low numbers of receptors, results in a massive increase in receptor expression (up to 45,000 sites per cell) with a K_d of 3–12 n*M*. Several cell lines (HL-60, K562, MT-2, and HeLa) also express MIP-1β receptors (313). In competition binding studies with ^{125}I-labeled RANTES, low numbers of MIP-1α receptors (K_d = 1.6 n*M*) are found on THP-1 cells and monocytes (262). Two cDNAs that presumably code for MIP-1α receptors were isolated and expressed in cell lines or frog oocytes (237, 314). Receptors for murine MIP-1α are present on murine T lymphocytes and the macrophage cell lines, CTLL-R8 and RAW 264.7 (K_d = 0.9–1.5 n*M*; 1200 sites per cell or less) (315).

Receptors for RANTES on monocytes and THP-1 cells were identified in two independent studies. Although the affinities are similar (K_d = 0.4–1.0 n*M*), the receptor numbers differ by 10-fold, i.e., approximately 700 (262) versus 5000 sites per cell (312). This discrepancy may result from the anomalous behavior of ^{125}I-labeled RANTES in binding experiments due to increased association with the cells, presumably through aggregation, when nonlabeled RANTES is added in excess (262, 314). RANTES receptors also bind MIP-1α and MCP-1 with high affinity (K_d = 1.6 and 6 n*M*, respectively) (262). Low-affinity interaction with MCP-1 (K_d = 100 n*M*), however, is also reported (312). Cross-desensitization experiments showing that stimulation of THP-1 cells with MCP-1 or MIP-1α prevents the response to RANTES suggest that all three CC chemokines interact with RANTES receptors (262).

Although several CC chemokines activate basophils and eosinophils, little information on receptors has been published. Desensitization experiments suggest the existence of multiple receptors and of distinct patterns of receptor usage (241). As shown by the $[Ca^{2+}]_i$ changes induced by sequential stimulation of basophils, eosinophils, or monocytes (Fig. 4), all three types of leukocytes respond to RANTES and MIP-1α, whereas only basophils and monocytes respond

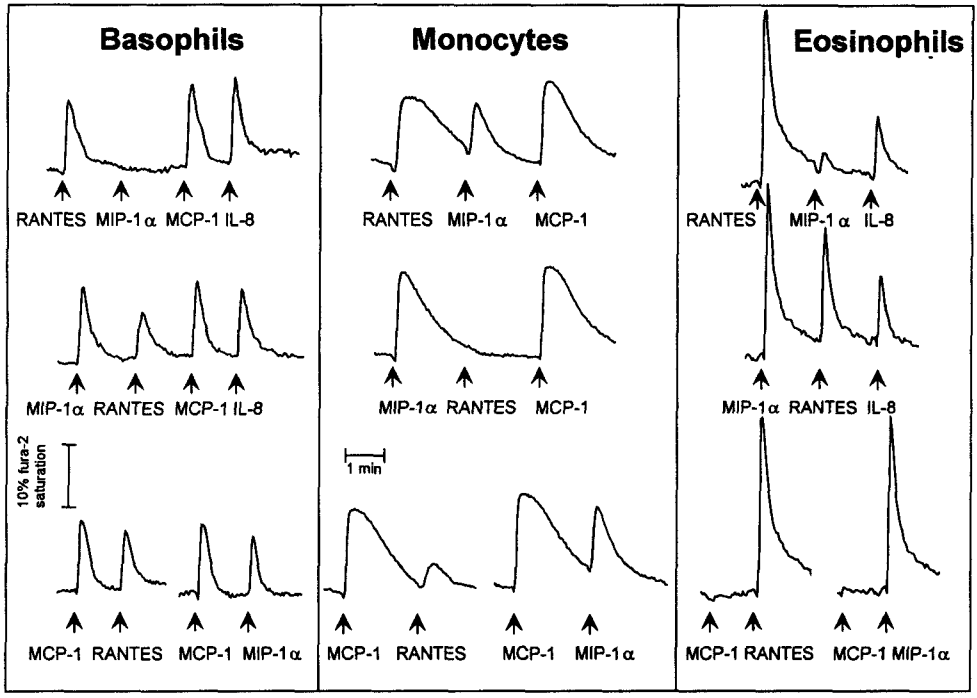


FIG. 4. Leukocyte responses to sequential stimulation with CC chemokines. Changes of the cytosolic free calcium concentration in highly purified basophils (>90%), eosinophils (>99%), and monocytes (>90%) stimulated with chemokines. Fura-2-loaded cells were suspended at 10^6 cells/ml and continuously monitored for fluorescence changes. Chemokines were added as indicated to a final concentration of 50 n*M* (basophils, eosinophils) and 5 n*M* (monocytes). Reproduced with permission from Bischoff *et al.* (241).

to MCP-1. In basophils and eosinophils stimulation with MCP-1 does not affect the $[Ca^{2+}]_i$ changes induced by a subsequent challenge with RANTES or MIP-1α, and no cross-desensitization is observed when the order of stimulation is reversed, indicating that basophils have distinct selective receptors for MCP-1 and for RANTES and MIP-1α. Mutual desensitization indicates that RANTES and MIP-1α share receptors. RANTES virtually abolishes the response to MIP-1α, whereas MIP-1α reduces the response to RANTES only slightly, suggesting the presence of a receptor for RANTES with little or no affinity for MIP-1α.

Responses to CC chemokines are quite different in monocytes, for which MCP-1, RANTES, and MIP-1α appear to share receptors, as

indicated by the desensitizing effect of MCP-1. RANTES and MIP-1α show again cross-desensitization, but in monocytes MIP-1α is more effective. A receptor for MIP-1α expressed in monocytes has recently been cloned (237, 314). It also binds MCP-1 and RANTES, which is in agreement with the results of desensitization experiments (314).

3. *Receptor on Erythrocytes*

A receptor for IL-8 (K_d = 5 n*M*; 2000 sites per cell) was characterized on erythrocytes by Darbonne *et al.* (316). This receptor is unique because it binds CXC and CC chemokines equally well. IL-8 is not internalized and can be rapidly displaced by GROα, NAP-2, MCP-1, and RANTES, but not by MIP-1α (317). Bound IL-8 is inactive toward neutrophils (316), but full activity is regained on displacement with another chemokine (T. Geiser and B. Dewald, unpublished observations). IL-8 binding to erythrocytes is frequently lacking in Afro-American individuals. A study of positive and negative blood samples revealed that the IL-8 receptor corresponds to the Duffy antigen. The malaria parasite, *Plasmodium vivax*, invades erythrocytes by binding to this antigen, and chemokines, GROα in particular, were found to prevent parasite recognition and invasion *in vitro* (R. Horuk, personal communication). It was suggested that erythrocytes, through this receptor, may function as scavengers of chemokines in plasma (316). High affinity anti-IL-8 antibodies as well as IL-8–IgG complexes are frequently found in the circulation of normal individuals, suggesting the existence of an alternative, efficient mechanism of IL-8 scavenging (318).

B. Receptor Cloning

A major step toward the understanding of neutrophil activation was the demonstration by Boulay and colleagues (319) that chemotactic agonist receptors have a seven-transmembrane-domain (7-TM) structure. Functional expression cloning or screening with degenerate oligonucleotide probes for conserved regions of G-protein-coupled receptors led to the identification of cDNAs for a rabbit IL-8 receptor (320, 321), two human IL-8 receptors (322, 323), human and murine receptors for C5a (324–326), several active and inactive isoforms of the human fMet-Leu-Phe receptor (327–331), the rabbit fMet-Leu-Phe receptor (332), and human and guinea pig receptors for platelet-activating factor (333, 334).

Extensive biological experimentation had indicated that the effects of IL-8 on neutrophils are typical for chemotactic agonists (1a, 210). The cloning and structural characterization of IL-8 receptors provided

TABLE IV
AMINO ACID SEQUENCE COMPARISON OF CXC AND CC CHEMOKINE RECEPTORS

	huIL-8R2	rbIL-8R	rbF3R	huMIP-1αR	huLESTR52	huMDCR15	US28C	US28N
huIL-8R1	77[a]	84	79	29	32	38	29	29
huIL-8R2		73	67	31	34	40	29	28
rbIL-8R			94	30	31	38	28	27
rbF3R				27	28	36	26	26
huMIP-αR					31	30	30	29
huLESTR52						34	29	29
huMDCR15							19	21
US28C								93

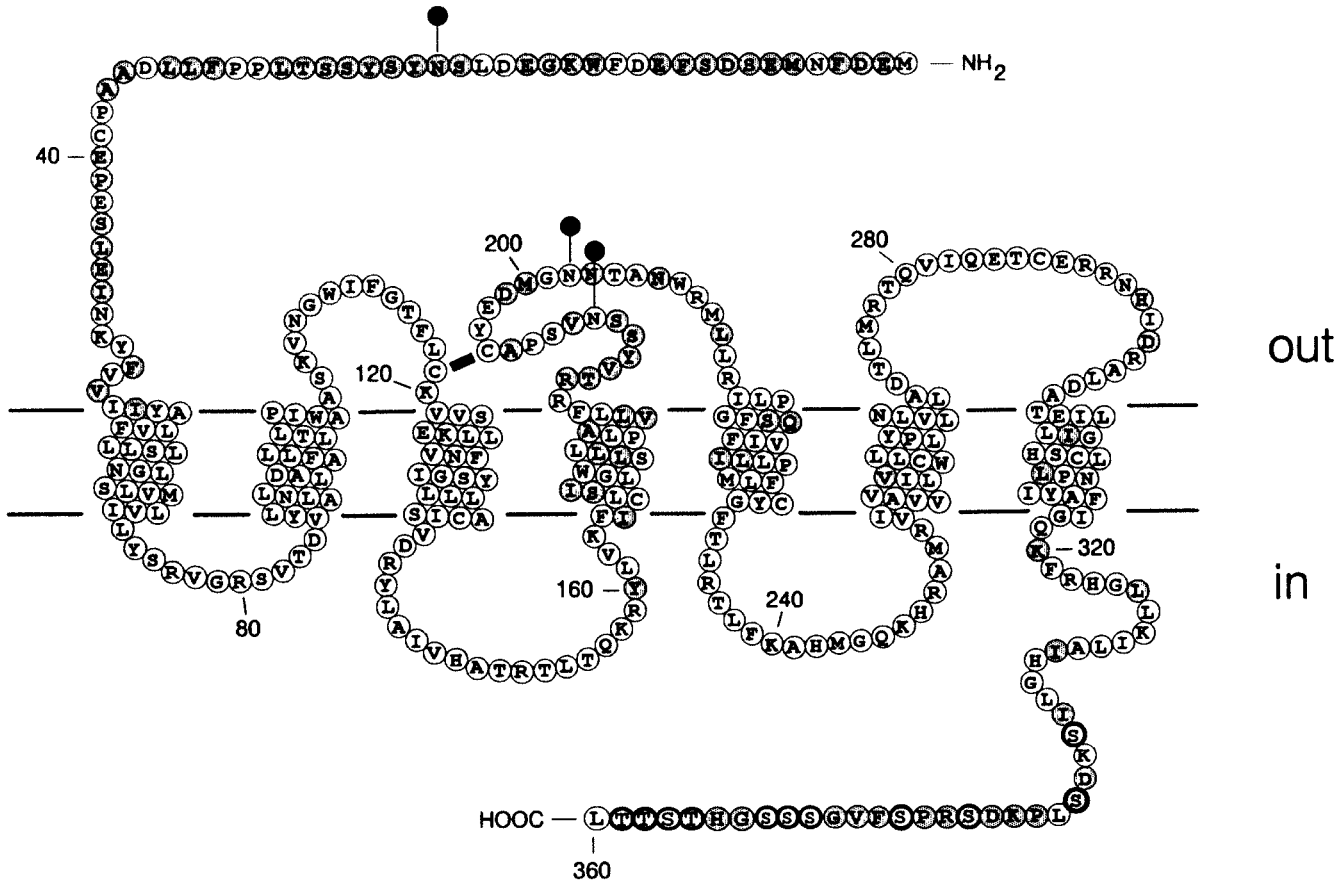
[a] Numbers refer to percentage amino acid identity. Pairwise receptor protein sequence alignments were carried out using the program PALIGN (414) and the Unitary Matrix (415) with an open gap cost and unit gap cost of 3 and 2, respectively.

the last piece of evidence that IL-8 and its related chemokines are primarily chemoattractants. The identification of two distinct cDNAs is in agreement with the biochemical evidence for the existence of two IL-8 receptors.

1. IL-8 Receptors

Two distinct human IL-8 receptors (IL-8R1 and IL-8R2) were cloned in 1991.[1] Holmes *et al.* (322) isolated a cDNA coding for a 350-amino acid protein (IL-8R1) from a human neutrophil cDNA library by expression screening for ^{125}I-labeled IL-8 binding in COS-7 cells. Murphy and Tiffany (323) isolated a cDNA coding for a 360-amino acid protein (IL-8R2) by screening of a cDNA library from dibutyryl cyclic AMP-differentiated HL-60 cells with a synthetic oligonucleotide probe for the rabbit IL-8 receptor, rbF3R, and expressed the functional receptor by cRNA microinjection in *Xenopus laevis* oocytes. The cDNAs for IL-8R1 and IL-8R2 code for polypeptides with M_r of approximately 40 kDa and a sequence identity of 77% (Table IV). Both sequences contain seven putative transmembrane domains of 20–25 amino acids with presumed α-helical structure, which are typical for G-protein-coupled receptors (Fig. 5). The N-terminal region and the second extracellular loop contain sites for N-linked glycosylation

[1] The receptor cloned by Holmes *et al.* (322) is most frequently termed IL-8R1, IL-8Rα, or IL-8A, and the one cloned by Murphy and Tiffany (323) is named IL-8R2, IL-8Rβ, or IL-8B. In our studies we called IL-8R1 the receptor that binds IL-8, GROα, and NAP-2 with high affinity, and IL-8R2 the other one (304, 307). In the present review, we adopt the common nomenclature: IL-8R1 for the IL-8-selective receptor, and IL-8R2 for the receptor with high affinity for IL-8, GROα, and NAP-2.


NH2
40
200
280
120
out
in
80
160
240
320
HOOC
360

that may account for the observed differences in the electrophoretic migration of the native receptor proteins as found in cross-linking experiments.

Both cDNAs were expressed in Jurkat, COS-7, and human embryonic 293 kidney cells, and the binding properties of the receptors were characterized. IL-8R1 binds IL-8 with high affinity and GROα and NAP-2 with low affinity, whereas IL-8R2 binds all three CXC chemokines with high affinity (269, 335, 336). These results confirm the functional characterization of two IL-8 receptors in human neutrophils (304, 307) (Fig. 3). Some uncertainty exists about the sequence of the rabbit IL-8 receptor (320, 321). Two identical cDNAs were independently isolated by PCR using primers derived from the published rabbit IL-8 receptor sequence (337, 338). The polypeptide encoded by these cDNAs differs from the rabbit IL-8 receptor, rbF3R (321), by 23 amino acids (Fig. 6). Since clones encoding rbF3R were not found, it is not clear whether the disagreement is due to the occurrence of receptor isoforms or to sequencing artifacts.

2. *Other Chemokine Receptors*

A high-affinity 7-TM receptor for MIP-1α (K_d = 1 n*M*) was recently cloned and expressed in E293 cells (314) and in *X. laevis* oocytes (237). The recombinant receptor binds MIP-1α with high affinity and MCP-1 with low affinity. It could not be ascertained whether ^{125}I-labeled RANTES binds to this MIP-1α receptor, but the displacement of ^{125}I-labeled MIP-1α by unlabeled RANTES and the Ca^{2+} mobilization experiments suggest that a selective interaction occurs (314). It was known that the putative G-protein-coupled receptor US28C, encoded by the human cytomegalovirus AD169, is strikingly similar to the chemokine receptors (339) (Fig. 6). US28N, a variant of US28C, binds MIP-1α with high affinity when expressed in E293 cells, and competition is observed with MCP-1, MIP-1β, and RANTES (314). Neither MIP-1αR nor US28N recognize IL-8. Sequence identities between MIP-1αR or the US28 polypeptides and the IL-8 receptors range between 26 and 31% (Table IV).

Two novel receptors of yet unidentified ligand specificity, LESTR52 and MDCR15, were found in our and several other laboratories. We

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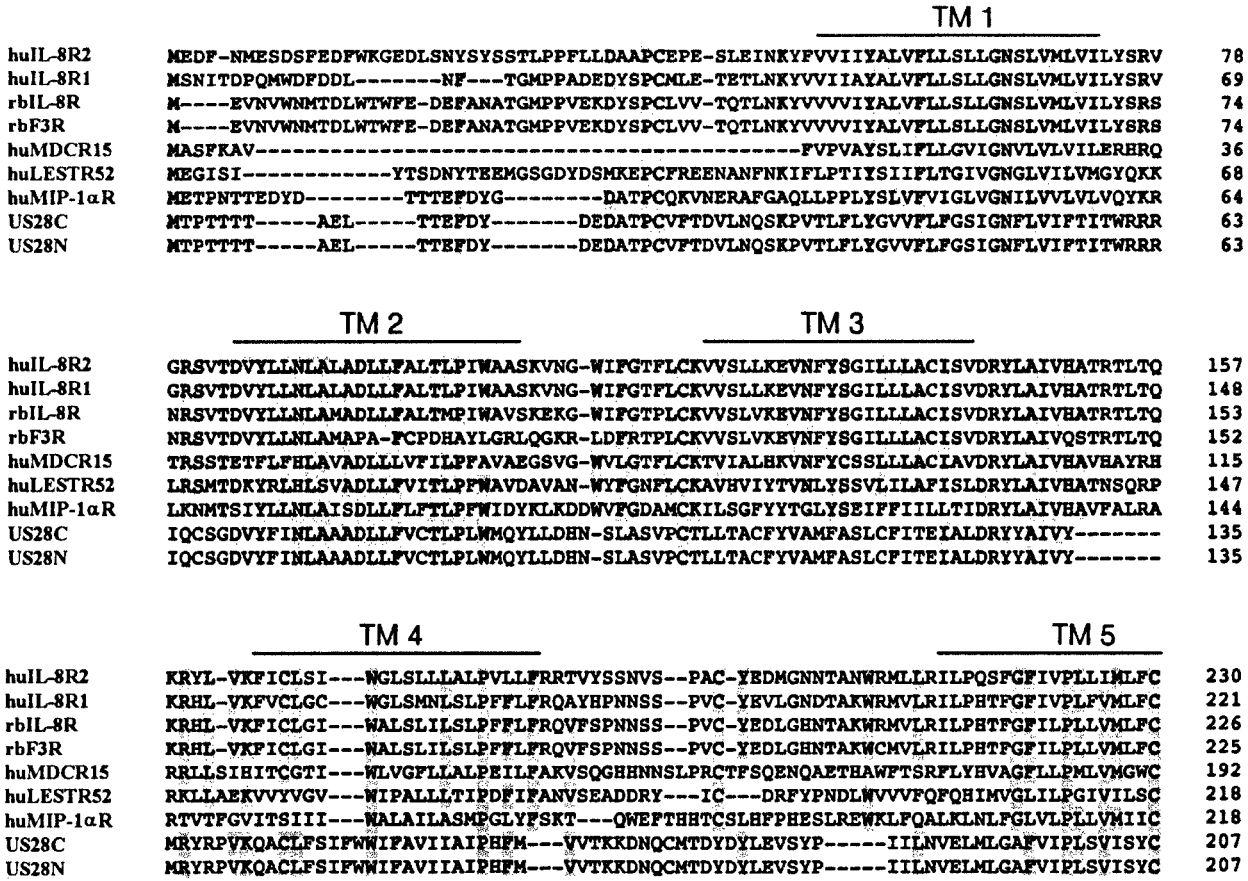
FIG. 5. Scheme of IL-8R2. Amino acids that differ from those of IL-8R1 are shaded. Note the extensive sequence identity between IL-8R1 and IL-8R2 in the region extending from transmembrane domain 1 to 7. Pinheads indicate putative N-linked glycosylation sites of IL-8R2, and bold circles highlight serines and threonines of the C-terminal domain as potential phosphorylation sites.


TM 1
TM 2
TM 3
TM 4
TM 5
huIL-8R2 MEDF-NMESDSFEDFWKGEDLSNYSYSSTLPPFLLDAAPCEPE-SLEINKYFVVIIYALVFLLSLLGNSLVMLVILYSRV 78
huIL-8R1 MSNITDPQMWDFDDL-------NF---TGMPPADEDYSPCMLE-TETLNKYVVIIAYALVFLLSLLGNSLVMLVILYSRV 69
rbIL-8R M----EVNVWNMTDLWTWFE-DEFANATGMPPVEKDYSPCLVV-TQTLNKYVVVVIYALVFLLSLLGNSLVMLVILYSRS 74
rbF3R M----EVNVWNMTDLWTWFE-DEFANATGMPPVEKDYSPCLVV-TQTLNKYVVVVIYALVFLLSLLGNSLVMLVILYSRS 74
huMDCR15 MASFKAV----------FVPVAYSLIFLLGVIGNVLVLVILERHRQ 36
huLESTR52 MEGISI----------YTSDNYTEEMGSGDYDSMKEPCFREENANFNKIFLPTIYSIIFLTGIVGNGLVILVMGYQKK 68
huMIP-1αR METPNTTEDYD--------TTTEFDYG--------DATPCQKVNERAFGAQLLPPLYSLVFVIGLVGNILVVLVLVQYKR 64
US28C MTPTTTT-----AEL-----TTEFDY-------DEDATPCVFTDVLNQSKPVTLFLYGVVFLFGSIGNFLVIFTITWRRR 63
US28N MTPTTTT-----AEL-----TTEFDY-------DEDATPCVFTDVLNQSKPVTLFLYGVVFLFGSIGNFLVIFTITWRRR 63
huIL-8R2 GRSVTDVYLLNLALADLLFALTLPIWAASKVNG-WIFGTFLCKVVSLLKEVNFYSGILLLACISVDRYLAIVHATRTLTQ 157
huIL-8R1 GRSVTDVYLLNLALADLLFALTLPIWAASKVNG-WIFGTFLCKVVSLLKEVNFYSGILLLACISVDRYLAIVHATRTLTQ 148
rbIL-8R NRSVTDVYLLNLAMADLLFALTMPIWAVSKEKG-WIFGTPLCKVVSLVKEVNFYSGILLLACISVDRYLAIVHATRTLTQ 153
rbF3R NRSVTDVYLLNLAMAPA-FCPDHAYLGRLQGKR-LDFRTPLCKVVSLVKEVNFYSGILLLACISVDRYLAIVQSTRTLTQ 152
huMDCR15 TRSSTETFLFHLAVADLLLVFILPFAVAEGSVG-WVLGTFLCKTVIALHKVNFYCSSLLLACIAVDRYLAIVHAVHAYRH 115
huLESTR52 LRSMTDKYRLHLSVADLLFVITLPFWAVDAVAN-WYFGNFLCKAVHVIYTVNLYSSVLILAFISLDRYLAIVHATNSQRP 147
huMIP-1αR LKNMTSIYLLNLAISDLLFLFTLPFWIDYKLKDDWVFGDAMCKILSGFYYTGLYSEIFFIILLTIDRYLAIVHAVFALRA 144
US28C IQCSGDVYFINLAAADLLFVCTLPLWMQYLLDHN-SLASVPCTLLTACFYVAMFASLCFITEIALDRYYAIVY------- 135
US28N IQCSGDVYFINLAAADLLFVCTLPLWMQYLLDHN-SLASVPCTLLTACFYVAMFASLCFITEIALDRYYAIVY------- 135
huIL-8R2 KRYL-VKFICLSI---WGLSLLLALPVLLFRRTVYSSNVS--PAC-YEDMGNNTANWRMLLRILPQSFGFIVPLLIMLFC 230
huIL-8R1 KRHL-VKFVCLGC---WGLSMNLSLPFFLFRQAYHPNNSS--PVC-YEVLGNDTAKWRMVLRILPHTFGFIVPLFVMLFC 221
rbIL-8R KRHL-VKFICLGI---WALSLILSLPFFLFRQVFSPNNSS--PVC-YEDLGHNTAKWRMVLRILPHTFGFILPLLVMLFC 226
rbF3R KRHL-VKFICLGI---WALSLILSLPFFLFRQVFSPNNSS--PVC-YEDLGHNTAKWCMVLRILPHTFGFILPLLVMLFC 225
huMDCR15 RRLLSIHITCGTI---WLVGFLLALPEILFAKVSQGHHNNSLPRCTFSQENQAETHAWFTSRFLYHVAGFLLPMLVMGWC 192
huLESTR52 RKLLAEKVVYVGV---WIPALLLTIPDFIFANVSEADDRY---IC---DRFYPNDLWVVVFQFQHIMVGLILPGIVILSC 218
huMIP-1αR RTVTFGVITSIII---WALAILASMPGLYFSKT---QWEFTHHTCSLHFPHESLREWKLFQALKLNLFGLVLPLLVMIIC 218
US28C MRYRPVKQACLFSIFWWIFAVIIAIPHFM---VVTKKDNQCMTDYDYLEVSYP-----IILNVELMLGAFVIPLSVISYC 207
US28N MRYRPVKQACLFSIFWWIFAVIIAIPHFM---VVTKKDNQCMTDYDYLEVSYP-----IILNVELMLGAFVIPLSVISYC 207

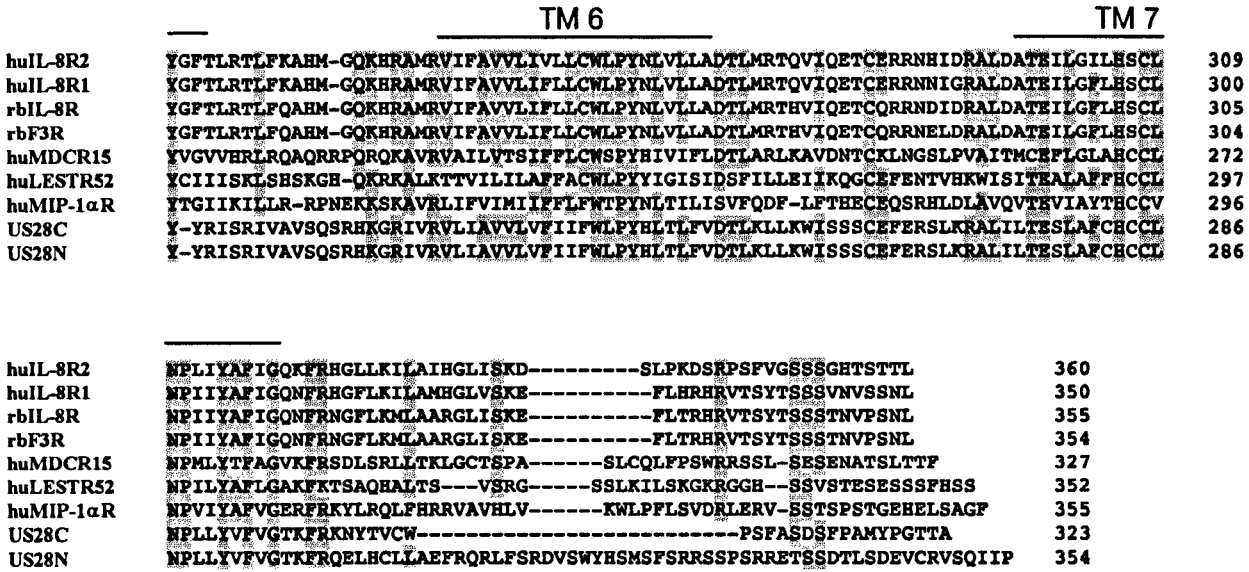


FIG. 6. Deduced amino acid sequences of chemokine receptors. Human IL-8R1 (322), human IL-8R2 (323), human MIP-1αR (237, 314), rabbit IL-8R (337, 338), rabbit F3R (320), the human cytomegalovirus-encoded proteins US28C (339) and US28N (314), and the putative human chemoattractant receptors MDCR15 (339b) and LESTR52 (339a) are shown. Shaded areas indicate extensive sequence homologies (six or more amino acid residue identities) and overbars denote the putative transmembrane domains (TM). Protein alignment using the program CLUSTAL according to Higgins and Sharp (414).

have isolated the cDNAs from a human monocyte cDNA library, and the recombinant polypeptides share striking similarities with the human IL-8 receptors (339a, 339b). An isoform of MDCR15, BRL1, which has an N-terminal extension of 45 amino acids due to RNA splicing, was cloned from a Burkitt's lymphoma cell line (340). Using PCR techniques we found that MDCR15 and BRL1 mRNAs are expressed at low levels in blood leukocytes. Expression is high, however, in Burkitt's lymphoma and human chronic lymphocytic leukemia cells (339b). It will be interesting to determine if alternative splicing is a mechanism for regulating expression and function of these receptors, which are more related to the IL-8 receptors than to the receptors for CC chemokines, MIP-1αR and US28N (Fig. 6, Table IV). LESTR52 is remarkable because of its astonishingly high level of expression in normal granulocytes and mononuclear phagocytes, as shown by Northern analysis and by the incidence of LESTR52-positive clones, which was 20-fold higher than that of IL-8R1 and IL-8R2 clones (339a). Neither LESTR52 nor MDCR15 appear to be expressed in normal tissue cells. Although no binding of chemokines, such as IL-8, GROα, NAP-2, IP10, PF4, MCP-1, RANTES, HC14, and I-309, could be detected, selective expression in white blood cells suggests that these receptors may interact with as yet unidentified CXC or CC chemokines or other leukocyte-activating agonists.

3. *Structural Similarities*

Like the receptors for fMet-Leu-Phe, C5a, and PAF, CXC and CC chemokine receptors are single-chain polypeptides of 350 to 360 amino acids (341) (Fig. 6). As compared to other 7-TM receptors, chemokine receptors have relatively short N- and C-terminal regions (Fig. 6). In particular, the third intracellular loops which do not exceed 20 amino acids are very small compared to the loops of more than 150 residues found in some adrenergic receptors (341). Some residues and motifs of the chemokine receptors are highly conserved among the entire family of G-protein-coupled receptors, such as the prolines in transmembrane domains 2, 4, 5, 6, and 7, which may be involved in the formation of the ligand-binding pocket, a tryptophan in transmembrane domains 4 and 6, the Gly-Asn-X-X-Val sequence in transmembrane domain 1, the Leu-Ala-X-Ala-Asp sequence in transmembrane domain 2, a phenylalanine in transmembrane domain 6, and an asparagine and a tyrosine in transmembrane domain 7 (Fig. 6). Also conserved is the Asp-Arg-Tyr motif in the second intracellular loop that was shown to be important for signal transduction (342–344). In the

chemokine receptors this motif is followed by a conserved sequence of four to six amino acids that may confer selectivity in signaling. Two highly conserved cysteines are present in the first and second extracellular loops. By analogy to the β-adrenergic receptor and rhodopsin, they are believed to form a disulfide bond that is essential for ligand binding (345, 346). Several cysteines are considered to be critical for stabilizing the tertiary structure of the membrane-spanning helices. Pro-Cys in the N-terminal region and the cysteine in the third extracellular loop are strictly conserved in all chemokine receptors.

4. Receptor Genes

The genes for the formyl-peptide receptor and its inactive isoforms, FPRH1 and FPRH2, have a similar genomic organization, and their products, like most G-protein-coupled receptors, are encoded by a single exon (347, 348). The C5a receptor gene, by contrast, contains two exons; the first contributes the 5′ untranslated region plus the translation start codon for methionine, and the second, approximately 9 kb downstream, codes for the rest of the receptor and the 3′ untranslated region (326, 347). These genes are tightly clustered at the interface of bands q13.3 and 13.4 of human chromosome 19 (326, 328, 347, 349). The genes for IL-8R1, IL-8R2, and the IL-8 pseudoreceptor, IL-8RP, were mapped to band q35 of chromosome 2 (349, 350), whereas the gene for MIP-1αR, the only CC chemokine receptor that has been cloned, is located on chromosome 3, p21 (237).

C. IL-8 Receptor Expression

Both IL-8 receptors (IL-8R1 and IL-8R2) are abundantly expressed in neutrophils, the primary target cells of CXC chemokines, but only low numbers are found on blood monocytes and lymphocytes (242, 256, 257, 268) (Table III). IL-8 receptors are also expressed by human promyelocytic and monocytic leukemia cell lines following differentiation toward granulocytes, but are not found in lymphoid cell lines of the T and B lineage, e.g., Jurkat, SKW3, and Raji cells, nor on cultured tissue cells, such as fibroblasts and epithelial cells. Receptor mRNA is detected only by RT-PCR in some of these cells, but receptor expression appears to be too low for detection in binding assays (269). IL-8 was reported to be chemotactic (281) and mitogenic (280) for melanoma cells, keratinocytes (285), and umbilical vein endothelial cells (284), but no information is available on receptors that mediate these effects. RT-PCR analysis indicates that some primary human melanoma cells express IL-8R1 but not IL-8R2 (269).

D. Structural Considerations

1. Binding of CXC Chemokines

The amino acid sequence identity between the two IL-8 receptors is high in the region extending from the first to the seventh transmembrane domain (87%), and low in the N-terminal region (29%) and the second extracellular loop (55%) (Fig. 5). Binding studies with cells expressing chimeric receptors made by exchanging the N-terminal domains of the human IL-8R2 and the rabbit F3R (which, like human IL-8R1, is IL-8 selective) show that high-affinity binding of GROα and NAP-2 to IL-8R2 depends on interaction with the N-terminal region (336). Several acidic residues in this region of IL-8R1 and IL-8R2 are likely to interact with positive charges of the cationic CXC chemokines. The binding of C5a also appears to depend on the N-terminal receptor domain, as shown by monoclonal antibodies that block discrete epitopes (O. Götze, personal communication) and by mutations (F. Boulay, personal communication). Extensive studies with fMet-Leu-Phe/C5a receptor chimeras, obtained by reciprocal substitutions of intra- and extracellular domains, suggest that all three extracellular loops are required for the binding of fMet-Leu-Phe (351). It cannot be excluded, however, that these substitutions affect ligand binding indirectly by altering receptor conformation. In contrast to the CXC chemokines, fMet-Leu-Phe shows little interaction with the N-terminal domain of its receptor (351).

2. The ELR Motif of CXC Chemokines

On the basis of three-dimensional analysis by NMR spectroscopy and X-ray crystallography it was suggested that IL-8 binds to its receptor through its C-terminal α-helix (352). Studies have shown, however, that the critical binding domain is at the N terminus (77, 353). Using chemically synthesized analogs, we have found that removal of the entire C-terminal helix decreases, but does not suppress, the biological activity of IL-8. In contrast, receptor binding and neutrophil activation are abrogated when the N-terminal sequence Glu-Leu-Arg (ELR) that precedes the first cysteine is deleted (77) (Fig. 7). All three residues, arginine in particular, are highly sensitive to modification (77, 354). The same conclusion was drawn from an elegant mutagenesis study by Hébert *et al.* (353), showing that IL-8 activity is lost when residues within the ELR motif are replaced.

The critical role of the N terminus for receptor binding and biological activity is underscored by the fact that the ELR motif is common to all CXC chemokines that activate and attract neutrophils, but is absent

				K_d (nM)	Activity (%)
IL-8		77	AVLPRSAK **E L R** CQCIKT...	0.35	20
		1-72	SAK **E L R** CQCIKT...	0.25	100
		3-72	K **E L R** CQCIKT...	0.18	300
	ELR-IL-8	4-72	**E L R** CQCIKT...	0.22	300
		5-72	**L R** CQCIKT...	0.76	3
		6-72	**R** CQCIKT...	50	<0.1
		7-72	CQCIKT...	>10,000	<0.1
PF4		1-70	EAEEDG **D L Q** CLCVKT...	>10,000	<0.1
	DLQ-PF4	7-70	**D L Q** CLCVKT...	>10,000	<0.1
	ELR-PF4	7-70	**E L R** CLCVKT...	4	20
					IC_{50} (μM)
IL-8 Antagonists	ELQ-IL-8	4-72	**E L Q** CQCIKT...	700	1.1
	ELL-IL-8	4-72	**E L L** CQCIKT...	1,300	0.7
	AAR-IL-8	4-72	**A A R** CQCIKT...	8	0.3
	IR-IL-8	5-72	**I R** CQCIKT...	10	0.3
	R-IL-8	6-72	**R** CQCIKT...	50	0.3

FIG. 7. N-terminal modifications of IL-8 and PF4. Shown are the N-terminal sequences through the first two cysteines in one-letter code; the ELR motif is in boldface type. The truncations are indicated by the numbers; 1 and 72 refer to the N- and C-terminal positions of the 72-amino acid form (1–72) of IL-8, and 1 and 70 refer to those of the 70-amino acid PF4. K_d values are determined in ^{125}I-labeled IL-8(1–72) competition binding experiments. In neutrophil elastase release experiments, activities of the variants are compared to IL-8(1–72) as standard (100%), and IC_{50} values refer to inhibition of IL-8(1–72) activity. Experimental details have been described (77, 357, 354).

in PF4 and IP10, which are devoid of such activities (34, 47) (Fig. 1). The length of the N-terminal sequence is also important, as shown by the marked increase in activity obtained on progressive truncation of IL-8 (77) and CTAP-III (35) (see Section II,C for more information on N-terminal processing). Single amino acids in the sequence preceding the ELR motif of CXC chemokines may also affect binding, as shown by comparison of the activities of natural and truncated forms of the three GRO proteins. Deletion of five N-terminal amino acids has little effect on the activity of GROα and GROγ, but strongly enhances the activity of GROβ. The lower potency of the natural form of GROβ may be due to the presence of a proline at position 2 (instead of serine), which could partially hinder the interaction with the receptor (44).

A direct demonstration that the ELR motif is essential for CXC chemokine activity on neutrophils was obtained by substituting ELR for the natural DLQ sequence in PF4 (Fig. 7). ELR-PF4 competes for IL-8 binding and induces chemotaxis and enzyme release responses in neutrophils similar to those observed with IL-8. On the other hand, the truncated form of PF4 (DLQ-PF4) and IL-8 with ELQ in place of ELR are inactive (354). The ELR sequence was also introduced in IP10 and MCP-1, which belong to the CXC and CC proteins, respectively. In

these instances, however, no activity on neutrophils was obtained, suggesting that additional domains of the CXC chemokine molecules are required for receptor interaction. This notion is supported by the fact that several linear and cyclic ELR-containing peptides do not bind to the IL-8 receptors or stimulate neutrophils (354). The decapeptide AVLPRSAKEL corresponding to the N-terminal region of the 77-residue form of IL-8, which was reported to activate neutrophils (355), was totally inactive in our hands (77). The substitution of Tyr-28 and Arg-30 to Leu and Val in MCP-1 was reported to lower the activity toward monocytes and to confer neutrophil chemotactic activity to the CC chemokine (356). Using the same MCP-1 analog prepared by automated chemical synthesis, we were unable to confirm these results (I. Clark-Lewis and B. Dewald, unpublished).

After recognizing the importance of the N terminus, a series of analogs of the truncated form of IL-8 with substitutions within the ELR motif were synthesized as potential IL-8 receptor antagonists. Several of a total of 26 analogs with deletions or amino acid replacements in the region preceding the first cysteine inhibit IL-8 function. The most potent antagonists are R-IL-8 and AAR-IL-8 (Fig. 7). They inhibit IL-8 receptor binding, exocytosis (IC_{50} 0.3 μM), as well as chemotaxis and the respiratory burst. Inhibition is restricted to responses elicited by IL-8, GROα, or NAP-2, and no effect is observed when the unrelated agonists fMet-Leu-Phe or C5a are used as stimuli, demonstrating that both antagonists are selective for IL-8 receptors (357).

Taken together, these results prove that the N-terminal tripeptide, ELR, is of primary importance for the binding of CXC chemokines to the IL-8 receptors. The N-terminal domain is conformationally disordered, but is anchored to the highly structured core of the protein (by the two disulfide bonds) that bears additional binding sites for the selective interaction with extracellular domains of the receptor. Such secondary interactions may facilitate the access of the ELR sequence to the ligand-binding pocket formed by the transmembrane helices.

3. *G-Protein Coupling*

The effector functions induced by agonists that activate 7-TM receptors depend on the type of G-protein that couples to the receptor. Knowledge of structural domains that promote the interaction with G-proteins is, therefore, vital to the understanding of chemokine signaling. For the α- and β-adrenergic receptors, recognition sites have been identified within the second and third intracellular loop and the proximal region of the C-terminal sequence (341–343). Higashijima *et al.* observed that mastoparan, a wasp venom peptide with amphipathic

helical structure, interacts with G-proteins in a receptor-like fashion (358). Using synthetic peptides that correspond to intracellular regions of rhodopsin, multiple interactions were shown to be required for activation of transducin (359). Extension of these studies led to the proposal of a consensus for G_i activation sequences comprising two or more basic residues that are separated by 10–26 amino acids from a C-terminal basic motif (360, 361). All chemokine receptors characterized so far contain one or more sequences that are similar to the proposed consensus. Their short third intracellular loop may function as a G-protein recognition site, although the sequences separating the basic residues consist of less than 10 amino acids (Table V).

4. Receptor Regulation

Leukocyte responses to chemoattractants are of short duration because of rapid receptor desensitization. "Homologous desensitization" occurs at high ligand concentrations through phosphorylation by specific receptor kinases, and is restricted to the type of receptor that has been activated, as shown for the β_2-adrenergic receptor kinases and rhodopsin kinase (362–364). Other mechanisms of regulation, such as receptor sequestration or internalization, and down-regulation of receptor gene expression are slow processes, and are thus unlikely to regulate the duration of the response. The kinases are activated at high ligand or photon concentration and phosphorylate multiple serines and/or threonines in the C-terminal region of ligand-bound receptors, leading to interaction with arrestins and inhibition of G-protein coupling (342, 365–367).

TABLE V
PUTATIVE SEQUENCES INVOLVED IN RECEPTOR–G_i PROTEIN COUPLING

Receptor	Third intracellular loop
huIL-8R1	F T L R T L F K A H M————G Q K H R A M R
huIL-8R2	F T L R T L F K A H M————G Q K H R A M R
rbIL-8R	F T L R T L F Q A H M————G Q K H R A M R
rbF3R	F T L R T L F Q A H M————G Q K H R A M R
huMDCR15	G V V H R L R Q A Q R R P ————Q R Q K A V R
huLESTR52	I I I S K L S H S K G H————————Q K R K A L K
huMIP-1αR	G I I K I L L R R ————P N E K K S K A V R
US28W	Y R I S R I V A V S Q S R H K G R I V R
US28N	Y R I S R I V A V S Q S R H K G R I V R
Consensus[a]	B-$(X)_{1-5}$-B-$(X)_{3-9}$-BXBXXB

[a] B designates a basic residue (Lys, Arg, His), and X stands for any other amino acid.

Chemokine receptors contain 7 to 15 potential phosphorylation sites in the C-terminal region (Fig. 6). A mRNA for a kinase related to the β-adrenergic receptor kinase is highly expressed in human leukocytes, suggesting that chemotactic receptors may be desensitized by the mechanism known to operate for rhodopsin and the adrenergic receptors (368). A second process, termed "heterologous desensitization," affects different types of receptors through kinases that become activated during signal transduction, such as protein kinase C (PKC) or cAMP-dependent protein kinase A (342, 365). Several putative substrate consensus sequences for PKC (369, 370) are found in the intracellular domains of chemokine receptors (Table VI). Evidence for chemoattractant-induced desensitization is provided by work with the recombinant receptors for fMet-Leu-Phe and C5a (371). Studies by Boulay and colleagues show that these receptors are rapidly phosphorylated (<3 minutes) following addition of the ligands or PMA, supporting the hypothesis of chemoattractant receptor regulation by protein kinases (372).

VII. Signal Transduction

Several elements of the signal transduction cascade and their role in the regulation of single neutrophil responses have been studied using IL-8, fMet-Leu-Phe, and C5a as stimuli (206, 225). Agonist–receptor interaction initiates a characteristic pattern of responses, including shape change and chemotaxis, exocytosis, and the respiratory burst.

TABLE VI
PUTATIVE PKC SUBSTRATE SEQUENCES IN CHEMOKINE RECEPTORS[a]

Receptor	First intracellular loop	Second intracellular loop	Third intracellular loop
huIL-8R1	SRXXRS	TRTXTXKR	TXRTXXK
huIL-8R2	SRXXRS	TRTXTXKR	TXRTXXK
rbIL-8R	SRSXRS	TRTXTXKRH	TXRT
rbF3R	SRSXRS	TRTXTXKRH	TXRT
huMDCR15	RHRXTRSST	RHRRXXS	
huLESTR52	KKXRSXTXK	TXSXRXRK	SKXSHSK
huMIP-1αR	KRXKXXTS	RXRTXT	KKSK
US28C	TXRRR		RXSRXXXXSXSRHKXR
US28N	TXRRR		RXSRXXXXSXSRHKXR

[a] X designates any amino acid residue other than Ser, Thr, Lys, and Arg.

Using fMet-Leu-Phe and its butoxycarbonyl derivative that acts as an antagonist, it was shown that the responses depend on the persistence of the ligand–receptor complex and are discontinued when the agonist is displaced (206).

IL-8-bound receptors interact with GTP-binding proteins of the G_i type (G-proteins), as originally suggested by the inhibition of neutrophil responses after pretreatment with *B. pertussis* toxin (210). Exposure of neutrophil plasma membranes to IL-8, GROα, or NAP-2 enhances GTPase activity and the high-affinity binding of radiolabeled GTPγS, demonstrating that G-proteins are activated on CXC chemokine binding to the IL-8 receptors (308). *Bordetella pertussis* toxin also prevents monocyte activation by MCP-1 (259), suggesting that the same class of G-proteins mediates the functional responses to CC chemokines as well.

The next step in the cascade involves the activation of a phosphatidylinositol-specific phospholipase C that generates two second messengers, 1,4,5-inositoltrisphosphate (IP_3) and diacylglycerol. IP_3 induces the release of Ca^{2+} from intracellular stores, leading to a transient rise in $[Ca^{2+}]_i$, while diacylglycerol remains associated with the membrane and activates protein kinase C (225). IL-8 induces the concentration-dependent formation of IP_3 in human neutrophils (373) and the consequent rise in $[Ca^{2+}]_i$ (73, 210, 373). IL-8 also stimulates phosphatidylinositol-4-phosphate kinase, which synthesizes phosphatidylinositol-4,5-bisphosphate, the source of IP_3 (374). Pretreatment of the cells with an inhibitor of phospholipase C, U-73122, prevents the formation of IP_3 and $[Ca^{2+}]_i$ changes in response to stimulation with IL-8 (373). The transient $[Ca^{2+}]_i$ rise reflects the release of Ca^{2+} from intracellular stores and the influx from the extracellular medium (375). A rise in $[Ca^{2+}]_i$ is essential for the induction of exocytosis and the respiratory burst. By blocking the influx it can be shown that release of intracellular Ca^{2+} is sufficient to satisfy this requirement (376).

Sozzani *et al.* (259, 377) reported that MCP-1, in contrast to fMet-Leu-Phe, does not enhance IP_3 production and the turnover of phosphatidylinositol-4,5-bisphosphate in monocytes, and induces a $[Ca^{2+}]_i$ rise that is totally dependent on influx through plasma membrane channels. Under similar conditions, however, $[Ca^{2+}]_i$ transients are observed in monocytes that are stimulated with MCP-1 immediately after the addition of an excess of EGTA to chelate extracellular Ca^{2+} (Padrines *et al.*, unpublished). The $[Ca^{2+}]_i$ changes induced in monocytes by MCP-1, MIP-1α, and RANTES are similar (241, 377) (Fig. 4), but no information is available on the effect of CC chemokines other than MCP-1 under conditions that prevent Ca^{2+} influx. Analysis of the

$[Ca^{2+}]_i$ changes was used to assess the responsiveness and receptor selectivity of basophil and eosinophil leukocytes to CC chemokines (see Section VI,A,2). A later event of the signal transduction cascade in neutrophils is the activation of protein kinase C, which is required for the induction of the respiratory burst but not for other responses (378). Smith *et al.* (373) have shown that the α and β form of protein kinase C translocate from the cytosol to the plasma membrane in IL-8-stimulated neutrophils.

VIII. Role in Pathology

A. Skin Diseases

We like to begin the discussion of the role of chemokines in pathology with skin inflammation, because psoriasis, as shown by the work of Schröder (379), was the first disease to be linked to overproduction of IL-8. Up to several nanograms of biologically active IL-8 per milligram of scale material are found in psoriatic lesions, i.e., 100- to 200-fold the control concentration found in heel callus scrapings (379). Being more selective for neutrophils and more resistant to inactivation than other chemoattractants, IL-8 is generally believed to mediate the formation of neutrophilic skin microabscesses that characterize psoriatic skin (1a). IL-8 expression is not uniform in lesional skin. Nickoloff *et al.* (380) reported the presence of IL-8 mRNA (together with transforming growth factor-β mRNA) in the epidermal roof of the lesions. Immunohistochemical staining for TNFα, considered to be an inducer of IL-8, is present in dermal macrophages (dendrocytes) and in some keratinocytes and intraepidermal Langerhans cells, but not in endothelial cells, mast cells, or dermal Langerhans cells (380). *In situ* hybridization with an antisense IL-8 RNA probe shows the expression of IL-8 in association with keratinocytes of the upper layers of lesional epidermis, but not in uninvolved skin of patients or healthy controls (381). Hybridization yields a spotty pattern with a strong positive reaction at papillar tips, and is exclusive for IL-8, since TNFα and IL-6 mRNA are not detectable (381). Immunohistochemistry was used in several laboratories with contrasting results. Nickoloff *et al.* (380) observed intense staining in the upper lesional epidermis and no staining in nonaffected skin. Using two different monoclonal antibodies raised against natural IL-8, Sticherling *et al.* (382) found positive intracellular staining in suprabasal keratinocytes of healthy skin in patients and controls and decreased or even absent immunoreactivity in the lesions. Positive staining, mainly in the basal cell layer, in healthy and lesional skin of

patients with psoriasis and palmoplantar pustulosis, was found by Anttila *et al.* (383), who used different monoclonal and polyclonal antibodies raised against recombinant IL-8. Intra- and intercellular staining can be distinguished, and the intercellular staining appears to be characteristic for the lesions and disappears with healing. Interestingly, strong intracellular positivity is regularly detected in the eccrine sweat glands and, to a lesser extent, in endothelial cells and in mononuclear cell infiltrates (383). IL-8 is also detected in the gland epithelial cells by *in situ* hybridization, and IL-8 is recovered in the sweat of healthy joggers (S. Reitamo and B. Dewald, unpublished results).

Although little doubt exists concerning the role of IL-8 in inflammatory conditions such as psoriasis and palmoplantar pustulosis, in some skin diseases, such as eczema and ichthyosis congenita, considerable amounts of biologically active IL-8 can be extracted from the scales (379), although no neutrophil infiltration is observed in the tissue. It has been suggested that IL-8 alone is not sufficient to elicit neutrophil emigration, and that factors inducing the expression of adhesion molecules on endothelial cells are required. A rapid and direct recruitment of neutrophils, however, is obtained on local injection of IL-8 *in vivo* (292, 295) (see Section V,G). Alternatively, the absence of neutrophils in tissues that contain IL-8 could be explained by the lack of IL-8 release from the cells, the presence of an inhibitor, or desensitization, but no evidence for these possibilities has been provided.

In addition to neutrophils, lymphocytes infiltrate psoriatic skin. IL-8 is considered by some investigators to be the potential attractant (380, 384), although so far no experimental evidence has been provided that IL-8 is chemotactic for human lymphocytes *in vivo* (273, 274). Other chemotactic proteins, however, are likely to be produced with IL-8, and alternative mechanisms of lymphocyte infiltration may soon emerge. Studies of skin lesions at the protein or mRNA level suggest that keratinocytes are the main source of IL-8 and of other chemokines as well (Table II). It has also been reported that keratinocytes proliferate in response to IL-8, which, in conjunction with other factors, may contribute to the disease-related hypertrophy (J.M. Schröder, personal communication).

B. Arthritis

Several independent studies document the occurrence of high levels of IL-8 in the synovial fluid of inflamed joints of patients with different forms of rheumatic diseases, osteoarthritis, and gout (127, 144, 385–388). The neutrophil chemotactic activity of fluid samples

does not necessarily correlate with the levels of IL-8 (144), suggesting that other chemotactic factors, including other CXC chemokines (27), are present. The highest IL-8 levels are generally found in seropositive rheumatoid arthritis (387, 389), wherein the concentration of IL-8 correlates with the rheumatoid factor serum titer (387) and the level of IL-6 (390). No significant relation, however, has been established between IL-8 and clinical parameters of disease such as erythrocyte sedimentation rate and serum levels of C-reactive protein (387).

Because synovial fluid IL-8 may be released by cells of the inflamed synovium (27, 145, 204, 391), activated chondrocytes (392–394), and by neutrophils and mononuclear phagocytes (Table II), we studied the production of IL-8 by blood and synovial fluid mononuclear phagocytes from patients with a variety of inflammatory rheumatic diseases. The highest rates of spontaneous and stimulated IL-8 production are observed in patients with seropositive rheumatoid arthritis, indicating that their monocytes are activated (127, 146). The release of IL-8 correlated with the titers of circulating IgM rheumatoid factor, but not with other criteria for disease activity, such as the Landsbury and the Ritchie index (389). IL-8 production by mononuclear cells is inhibited by glucocorticoids, IFN-γ, gold salts, and methotrexate at clinically relevant concentrations, but not by indomethacin and other cyclooxygenase inhibitors (127, 389). Accordingly, intraarticular administration of glucocorticoids blocks the production of IL-8 by synovial fluid mononuclear cells (127).

In the same context, Koch *et al.* (395) studied the production of MCP-1. The concentration of MCP-1 is higher in the synovial fluid and plasma of patients with rheumatoid arthritis, as compared to other arthritic diseases, and is positively correlated with the concentration of IL-8. Although synovial fibroblasts (391, 395) and chondrocytes (185) can produce MCP-1, the main source is macrophages, which constitutively express this CC chemokine (395).

C. Lung Diseases

The existence of tissue-derived chemoattractants was proposed about 10 years prior to the discovery of IL-8 (396). Alveolar macrophages were recognized as a source of neutrophil chemotactic factors (397–399) that were believed to induce lung inflammation (397) and to enhance the microbicidal activity of neutrophils and lung defense against infection (400, 401).

Lung diseases with high neutrophil influx are at the center of interest. The increased expression of IL-8 mRNA in alveolar macrophages of patients with idiopathic pulmonary fibrosis correlates with the

amount of IL-8 and the number of neutrophils recovered by bronchoalveolar lavage and the severity of the disease (143), suggesting that IL-8 is a main causative agent of the neutrophilic alveolitis (402). Using *in situ* hybridization and immunocytochemistry, Antoniades *et al.* (150) showed that MCP-1 is also expressed by macrophages, as well as by epithelial, endothelial, and vascular smooth muscle cells in the lung of these patients. The MCP-1-dependent influx of mononuclear phagocytes is considered a prerequisite for the increased local synthesis of growth factors for mesenchymal cells that lead to fibrosis. High levels of IL-8 are found in acute inflammatory conditions of the lung, as in the adult respiratory distress syndrome, in which IL-8 and neutrophil numbers are reported to correlate with mortality (403), and in empyema (404). In cystic fibrosis, neutrophil elastase present in the lung epithelial lining fluid induces IL-8 production by bronchial epithelial cells (179). This process, which tends to amplify inflammation, can be restrained *in vivo* by aerosol application of "secretory leukoprotease inhibitor," a natural protein that blocks elastase and therefore IL-8 induction (405). Chemokines are also considered as potential mediators of inflammation in allergic diseases. Enhanced coexpression of IL-8 and GM-CSF is observed in bronchial epithelial cells of patients with asthma (406), a finding of interest since GM-CSF, IL-3, and IL-5 have been shown to enhance the responsiveness of basophils and eosinophils to CXC and CC chemokines (240, 241).

Several animal models have been used to assess the potential role of CXC and CC chemokines in lung pathology. Boylan *et al.* (407) showed that the acute pleurisy induced by intrapleural instillation of crocidolite asbestos in rabbits results from the induction of IL-8 formation by mesothelial cells in response to direct stimulation by asbestos. The rat alveolitis induced by IgA immune complexes is characterized by mononuclear phagocyte-dependent lung injury. Intravenous infusion of neutralizing antibodies against rat MCP-1 at the onset of immune complex alveolitis markedly reduces monocyte/macrophage accumulation, and attenuates increased vascular permeability and hemorrhage in the lung (408). The role of CXC chemokines for the recruitment of neutrophils in the rat lung is suggested by the presence of the mRNAs for two chemokines related to the human GRO proteins, MIP-2 and KC, in the bronchoalveolar lavage cells and trachea homogenate after instillation of LPS into the airways (409). An elegant demonstration of the involvement of MCP-1 in pulmonary granulomatosis was presented by Jones and Warren (410), who showed that the formation of angiocentric granulomata on intravenous injection of yeast cell wall glucan is characterized by monocytosis and the local accumu-

lation of mononuclear phagocytes. These changes depend on increased expression of MCP-1 mRNA in the lung and increased MCP-1 concentration in the bronchoalveolar lavage fluid.

D. Cardiovascular System

Two aspects of the potential involvement of CXC and CC chemokines in cardiovascular pathology are also worth considering—the formation of NAP-2 in thrombosis and the role of MCP-1 in atherosclerosis.

NAP-2 is the only neutrophil-activating chemokine that is not induced by gene activation, but is generated by proteolysis from the inactive precursors PBP and CTAP-III, which are released in bulk by aggregating platelets (35, 76). Cathepsin G and other serine proteases from monocytes convert the precursors into a neutrophil-attracting chemokine (35). NAP-2 is likely to be formed on platelet activation following endothelial damage and thrombosis. NAP-2-dependent accumulation of neutrophils may be important for the recanalization of thrombotic vessels (411).

MCP-1 and other CC chemokines are the subject of many studies because of their potential role in the recruitment of monocytes into atherosclerotic areas. Northern blot and *in situ* hybridization analyses, as well as immunohistochemistry, demonstrate enhanced expression and release of MCP-1 in macrophage-rich arterial wall areas in humans (151, 152) and in macrophage-derived foam cells in the lesions induced by a balloon catheter in cholesterol-fed rabbits (151). MCP-1, in contrast, is not detected in sublesional smooth muscle cells and in normal arterial tissue (151), although immunohistochemistry indicates that smooth muscle cells and the mesenchymal cells of the intima are capable of releasing this chemokine (152). MCP-1 production is also observed in the smooth muscle cells of the medial layer of the arterial wall and in monocytes and smooth muscle-like cells overlying intimal lesions in primates with diet-induced hypercholesteremia (412). Possible mechanisms for the recruitment of monocytes into arterial walls were studied in cocultures of endothelial and smooth muscle cells from human arteries (413). Low-density lipoprotein (LDL) added to these cultures is apparently modified by oxidation and acts as a stimulus for the expression of adhesion molecules and the production of MCP-1 by the endothelial cells, resulting in increased adherence and migration of monocytes across the endothelial layer. When LDL is added together with high-density lipoprotein, the modification is prevented and so is monocyte transmigration (413).

TABLE VII
EXPRESSION OF CXC AND CC CHEMOKINES IN DISEASE

System	Condition	Chemokine	Ref.
Joints	Gouty arthritis	IL-8	385
	Rheumatoid arthritis, osteoarthritis	IL-8	127, 144–146, 386–390, 444, 445
	Rheumatoid arthritis	MCP-1	184, 395
Skin	Contact dermatitis	IL-8	384, 446
	Cutaneous T cell lymphoma	IL-8	447–449
	Palmoplantar pustulosis	IL-8	383
	Psoriasis	IL-8	380–383
Lung	Adult respiratory distress syndrome	IL-8	403
	Asthma	IL-8	406
	Cystic fibrosis	IL-8	405
	Idiopathic pulmonary fibrosis	IL-8	143, 402
	Idiopathic pulmonary fibrosis	MCP-1	150
	Pleural empyema	IL-8	404
Various	Atherosclerosis	MCP-1	149, 151, 152, 413
	Bladder inflammation	IL-8	450
	Kawasaki disease	IL-8	451
	Ocular inflammation	IL-8	452
	Pancreatitis	IL-8	453
	Pertussis	IL-8	454
	Pregnancy, parturition	IL-8	455–458
	Red cell incompatibility	IL-8	459
	Relapsing fever	IL-8	460
	Sepsis, endotoxemia	IL-8	461–463
	β-Thalassemia	IL-8	464
	Transplantation	IL-8	464, 465
	Ulcerative colitis	IL-8	466, 467
	Uremia	IL-8	468
Animal model	Atherosclerosis	MCP-1	412
	Bacteremia	IL-8	469
	Lung inflammation	GRO	409
	Lung granulomatosis, alveolitis	MCP-1	408, 410
	Pleurisy	IL-8	407
	Renal ischemia	GRO	470
	Renal ischemia	MCP-1	470
	Wound healing	MIP-1, GRO	471

E. Other Conditions

Numerous publications during the past 2 years have demonstrated the expression of CXC and CC chemokines in pathological tissues. Many of these studies suggest that chemokines are the main attractants for inflammatory cells into the affected sites, but more work is required to assess their actual pathogenetic role. It is interesting to note that CXC and CC chemokines are often expressed concomitantly. An exhaustive list of reports is presented in Table VII.

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Receptors for Transforming Growth Factor-β

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I. Introduction

Transforming growth factor-β (TGF-β) was initially purified from human platelets as a 25-kDa homodimeric peptide using the ability of the factor to confer morphological transformation and anchorage-independent growth of normal rat kidney (NRK) fibroblasts as an assay (Assoian *et al.*, 1983). However, TGF-β was later found to be a potent growth inhibitor for many cell types (Moses *et al.*, 1985), and subsequent studies have revealed that TGF-β is a pleiotropic factor produced by many different cell types, including endothelial cells, smooth muscle cells, megakaryocytes, activated lymphocytes, and activated monocytes (reviewed in Roberts and Sporn, 1990; Massagué, 1990; Moses *et al.*, 1990). The biological effects that are mediated by TGF-β include regulation of cell proliferation, differentiation, adhesion, and migration; stimulation of extracellular matrix (ECM) deposition; as well as modulation of many inflammatory and immune responses. TGF-β has been proposed to have important *in vivo* physiological roles in tissue recycling, wound repair, and morphogenesis during embryogenic development (Roberts and Sporn, 1990).

Three distinct TGF-β isoforms have been identified in mammals; denoted TGF-β1 (Derynck *et al.*, 1985), TGF-β2 (de Martin *et al.*, 1987), and TGF-β3 (ten Dijke *et al.*, 1988; Derynck *et al.*, 1988), and they exert similar, but not identical, biological activities *in vitro* (Ohta *et al.*, 1987; Cheifetz *et al.*, 1990) as well as *in vivo* (Joyce *et al.*, 1990). Heterodimers have also been demonstrated in certain cell types, i.e., TGF-β1.2 in porcine platelets (Cheifetz *et al.*, 1987) and TGF-β1.2 and TGF-β2.3 in bovine bone (Ogawa *et al.*, 1992). Each of the TGF-βs is produced as a ~400-amino acid glycosylated precursor protein, which is proteolytically processed inside the cells into a C-terminal 112-amino acid mature peptide. The mature parts of the TGF-β isoforms have 70–80% sequence identity with a strict conservation of all nine cysteine residues, and each of the isoforms is highly conserved thoughout evolution (Derynck *et al.*, 1986; Miller *et al.*, 1989a,b). Each isoform is controlled by unique gene regulatory promoter/enhancer

elements (Roberts *et al.*, 1991) and their expression is strictly regulated in the tissues, e.g., during the development of heart and palate tissues (Millan *et al.*, 1991; Pelton *et al.*, 1991). These observations suggest that each TGF-β isoform has distinct functions *in vivo*.

The action of TGF-β is carefully controlled. TGF-βs are secreted from cells as latent forms that are activated under certain conditions (Pircher *et al.*, 1984; Wakefield *et al.*, 1987). In order to bind to specific cell surface receptors, TGF-β must be activated and released from the latent complex. Activation can be achieved efficiently by treatments with extreme pH values or heating (Brown *et al.*, 1990). In the latent TGF-β complex, the mature TGF-β remains noncovalently associated with the N-terminal remnant of the TGF-β precursor. Since this interaction renders TGF-β latent (Gentry *et al.*, 1987), the N-terminal remnant has been denoted the TGF-β latency-associated peptide (β-LAP). The latent TGF-β1 complex in human platelets was found to contain an additional component, called the latent TGF-β1 binding protein (LTBP) (Miyazono *et al.*, 1988; Wakefield *et al.*, 1988; Okada *et al.*, 1989; Kanzaki *et al.*, 1990; Tsuji *et al.*, 1990). Although the exact mechanism for activation of latent TGF-β *in vivo* remains to be elucidated, it most likely involves enzymatic degradation of β-LAP (reviewed in Miyazono and Heldin, 1991). LTBP is not needed for latency and its role in the complex is not fully understood. A possible function of LTBP has been suggested by studies of cocultures of endothelial and smooth muscle cells. TGF-β produced by these cells is activated only in the presence of both cell types, and activated TGF-β can be assayed directly by the inhibition of migration of endothelial cells (Sato and Rifkin, 1989). In this assay, LTBP was shown to mediate interactions, maybe at the cell surface, which appeared to be important for the activation of the latent TGF-β (Flaumenhaft *et al.*, 1993).

The cellular action of TGF-β is mediated through binding to cell surface receptors (Massagué, 1992; Lin and Lodish, 1993). Affinity labeling and cross-linking of radiolabeled TGF-β to cell surface proteins have revealed the existence in most cells of three distinct receptor types, termed receptors type I (53 kDa), type II (80 kDa), and type III (or betaglycan; 300-kDa proteoglycan). Our understanding of the signal transduction through TGF-β receptors has been dramatically advanced by the recent cloning and characterization of the TGF-β receptors type II (Lin *et al.*, 1992), and type III (López-Casillas *et al.*, 1991; Wang *et al.*, 1991). The different TGF-β receptor components appear to interact with each other in heteromeric receptor complexes. Recent studies have also shown that biologically active TGF-β interacts with various soluble proteins, which may serve as

reservoirs of TGF-β or as scavengers of excess TGF-β. This review focuses on the mechanisms of action of TGF-βs and related factors, and in particular on their interaction with specific receptors and binding proteins.

II. Structure and Activity of Members of the TGF-β Superfamily

A. Structure of TGF-β

The three-dimensional (3-D) structure of TGF-β2 was recently determined by X-ray crystallography at 2.1 and 2.2 Å resolution (Daopin *et al.*, 1992; Schlunegger and Grütter, 1992). Each chain of TGF-β2 consists of two pairs of antiparallel β sheets and three α helices (Fig. 1). Among the nine cysteine residues that are conserved in all TGF-β isoforms, eight are used for intrachain disulfide bonds, and one (Cys-77) forms an interchain disulfide bridge that stabilizes the dimeric

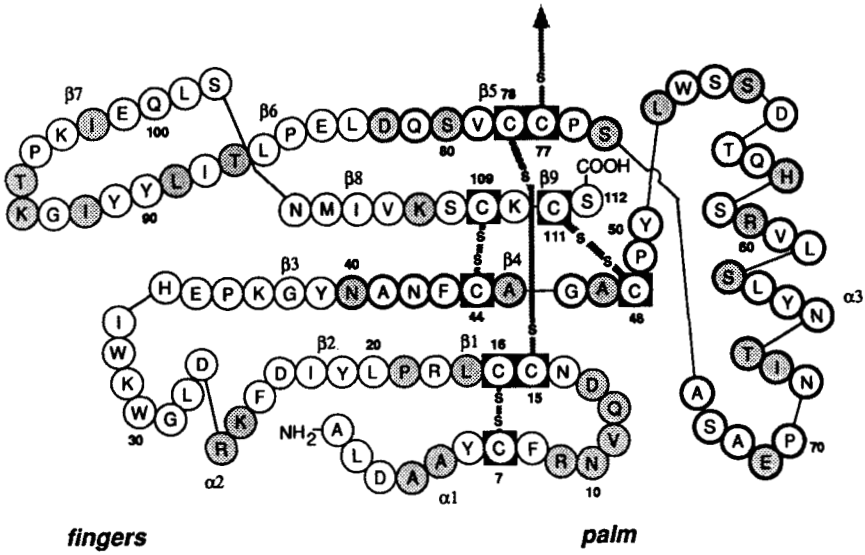


FIG. 1. Schematic illustration of the "hand-shaped" structure of TGF-β2. Amino acid residues are indicated by the one-letter code and are numbered from the N terminus of the mature molecule. Cysteine (C) residues are indicated by boxes. Amino acid residues that are different from those in TGF-β1 are shaded. Disulfide bonds are shown by thick lines (with S–S). Cys-77 forms an interchain disulfide linkage and stabilizes the dimer. Amino acid residues 40–82, which are important for the difference in bioactivity between TGF-β1 and TGF-β2 (Qian *et al.*, 1992), are indicated by thick lines encircling the one-letter code. The three α helices and nine β sheets are indicated as α1–α3 and β1–β9, respectively. Modified from Schlunegger and Grütter (1992) and Daopin *et al.* (1992).

structure. Instead of forming a compact globular protein, the TGF-β2 monomer has a structure that looks like a stretched, slightly curled hand with fingers. The intrachain disulfide bonds, together with hydrogen bonds, form a core structure (palm of the hand). In the dimeric structure, the heel of one hand attaches to the fingers of the other, with hydrophobic amino acids in the interface. The TGF-β1 sequence fits very well to the TGF-β2 backbone without any major distortion of the 3-D structure (Daopin *et al.*, 1992; Schlunegger and Grütter, 1992).

Although most of the biological activities are shared between TGF-β1, TGF-β2, and TGF-β3, there are some differences. TGF-β1 and TGF-β3 elicit their effects at ~100-fold lower protein concentrations compared to TGF-β2 on certain cell types, e.g., endothelial cells (Jennings *et al.*, 1988; Cheifetz *et al.*, 1990). Studies on a chimeric molecule of TGF-β1 and TGF-β2 revealed that the different responsiveness of endothelial cells to TGF-β1 and TGF-β2 can be assigned to amino acids 40–82 (Qian *et al.*, 1992). Fourteen amino acids are different between TGF-β1 and TGF-β2 in this region (Fig. 1); most of them are located at the surface of the molecule in the heel in the 3-D structure, which thus may be an important area for receptor binding (Daopin *et al.*, 1992). The availability of the 3-D structure of TGF-β2 will help elucidate the modes of interaction between TGF-βs and the different types of TGF-β receptors (and binding proteins).

B. TGF-β Superfamily

TGF-βs belong to a larger protein family referred to as the TGF-β superfamily, which contains proteins that are distantly related to TGF-βs in their structures (~30% sequence identity). The family includes activins and inhibins (reviewed in Vale *et al.*, 1990), bone morphogenetic proteins (BMPs) (Wozney *et al.*, 1988), Müllerian inhibiting substance (MIS) (Cate *et al.*, 1986), and certain other proteins involved in regulation of the development. The proteins in the TGF-β superfamily have seven invariant cysteine residues in each chain, but most of them lack the Cys-7 and Cys-16 of the TGF-βs, which form an intrachain disulfide bridge in TGF-β2 (Daopin *et al.*, 1992) (Fig. 1).

Activin was originally identified as a factor that induced the secretion of follicle-stimulating hormone (FSH) from the pituitary gland (Vale *et al.*, 1986; Ling *et al.*, 1986). Two homologous subunits of activin (β_A and β_B chains) form disulfide-bonded homodimers (activin A and activin B) and a heterodimer (activin AB). Activins have been shown to have additional effects, e.g., activin A stimulates the differentiation of hematopoietic progenitor cells into erythroid cells (Eto *et al.*, 1987; Murata *et al.*, 1988) and induces mesoderm formation in *Xenopus* embryos (Smith *et al.*, 1990; van den Eijnden-Van Raaij *et al.*,

1990). Inhibins are antagonists of activins and inhibit the pituitary secretion of FSH (Mason *et al.*, 1985). Inhibins are heterodimers of one α chain and one β chain (β_A or β_B chain). The β chains are shared between activins and inhibins; thus, the presence of the α chain leads to the exertion of completely opposite functions. Inhibin α chain was recently identified as a tumor suppressor gene based on the demonstration that a null mutation in mice for inhibin α chain, obtained by homologous recombination, led to the appearance of tumors in the gonadal stroma (Matzuk *et al.*, 1992). This indicates that inhibin is an important inhibitor of stromal cell proliferation in the gonads.

BMPs induce the formation of bone and cartilage when implanted subcutaneously (Wozney *et al.*, 1988). There are several different isoforms of BMPs, i.e., BMP-2, BMP-3 (or osteogenin), BMP-4 [or BMP-2b (Wozney *et al.*, 1988)], BMP-5 (Celeste *et al.*, 1990), Vgr-1 [or BMP-6, mammalian homologue of *Xenopus* Vg-1 (Weeks and Melton, 1987; Lyons *et al.*, 1989)], osteogenic protein (OP)-1 [or BMP-7 (Özkaynak *et al.*, 1990; Celeste *et al.*, 1990)], OP-2 (Özkaynak *et al.*, 1992), and Vgr-2 (Jones *et al.*, 1992). A heterodimer of BMP-2 and OP-1 was also purified from bovine bone (Sampath *et al.*, 1990). In addition to the effects on bone and cartilage, BMPs also have other effects; they facilitate neuronal differentiation (Paralkar *et al.*, 1992), induce monocyte chemotaxis (Cunningham *et al.*, 1992), and inhibit limb growth from the apical ectodermal ridge (Niswander and Martin, 1993). The products of the decapentaplegic (DPP) gene (Padgett *et al.*, 1987) and *60A* gene (Wharton *et al.*, 1991) of *Drosophila*, murine growth/differentiation factor (GDF)-1 (Lee, 1990), and the *Nodal* product (Zhou *et al.*, 1993) are structurally more similar to BMPs than to other members in the TGF-β superfamily, and thus form a subfamily together with the BMPs.

MIS induces regression of the Müllerian duct in the male reproductive system. MIS is the most distantly related member in the TGF-β superfamily. The active forms of most of the members of the TGF-β superfamily are dimers of the C-terminal parts of the precursor peptides, but the active form of MIS contains both the N-terminal (corresponding to β-LAP in TGF-β) and the C-terminal (corresponding to mature TGF-β) parts (Pepinsky *et al.*, 1988).

C. Bioactivity of TGF-β

1. Bifunctional Activity of TGF-β

The biological activities of TGF-β are often bidirectional (Roberts and Sporn, 1990). TGF-β stimulates the growth of certain mesenchymal cells, e.g., human fibroblasts and smooth muscle cells (Roberts *et*

al., 1985), but acts as a potent growth inhibitor for most cell types, e.g., endothelial cells (Baird and Durkin, 1986; Fràter-Schröder *et al.*, 1986), epithelial cells (Moses *et al.*, 1985), hematopoietic cells (Ohta *et al.*, 1987), and lymphocytes (Kehrl *et al.*, 1986a,b). TGF-β is chemotactic for fibroblasts (Postlethwaite *et al.*, 1987), monocytes (Wahl *et al.*, 1987), and neutrophils (Brandes *et al.*, 1991a), but inhibits the migration of endothelial cells (Heimark *et al.*, 1986). The effect of TGF-β on cellular differentiation is also dependent on the cell types; e.g., the differentiation of bronchial epithelial cells is induced by TGF-β (Masui *et al.*, 1986), but that of myoblasts is inhibited (Massagué *et al.*, 1986; Olson *et al.*, 1986; Florini *et al.*, 1986).

2. *ECM Production*

An important effect of TGF-β on target cells is the potent stimulation of ECM formation. TGF-β stimulates the production of ECM proteins, e.g., various types of collagens and fibronectin (Ignotz and Massagué, 1986). Moreover, there are two mechanisms by which TGF-β decreases the activity of pericellular proteases that act on ECM: (1) inhibition of the production of plasminogen activator (Laiho *et al.*, 1986), type I collagenase, and metalloproteinases (Edwards *et al.*, 1987), and (2) stimulation of the production of protease inhibitors, e.g., plasminogen activator inhibitor (PAI)-I (Laiho *et al.*, 1987) and tissue inhibitor of metalloproteinases (TIMP) (Edwards *et al.*, 1987). There are some exceptions to these two mechanisms, e.g., induction of type IV collagenase by TGF-β (Overall *et al.*, 1991; Salo *et al.*, 1991). Thus, TGF-β efficiently stimulates the accumulation of ECM proteins. TGF-β also induces the production of cell surface receptors for ECMs, e.g., integrins (Ignotz and Massagué, 1987; Heino and Massagué, 1989), and thereby promotes the interaction between cells and the ECM. TGF-β stimulates the angiogenesis *in vivo*, which may be due to the cellular migration and ECM deposition (Roberts *et al.*, 1986; Yang and Moses, 1990). When injected locally, TGF-β induces the formation of granulation tissue and angiogenesis (Roberts *et al.*, 1986) and accelerates wound healing (Mustoe *et al.*, 1987).

3. *Hematopoietic Cells*

TGF-β inhibits the growth of primitive hematopoietic progenitor cells, e.g., the interleukin (IL)-3-induced multipotent colony formation (Ohta *et al.*, 1987; Keller *et al.*, 1988; Hampson *et al.*, 1988), erythroid colony formation (Hino *et al.*, 1988; Ottmann and Pelus, 1988), and megakaryocytic colony formation (Ishibashi *et al.*, 1987). Granulopoiesis is, on the other hand, stimulated by TGF-β in the presence of granulocyte–macrophage colony-stimulating factor (GM-

CSF) (Ottmann and Pelus, 1988; Keller *et al.*, 1991). TGF-β was shown to inhibit the growth of myeloid leukemic cells and to induce their apoptotic cell death (Lotem and Sachs, 1992).

4. *Lymphocytes*

TGF-β is a potent regulator of the immune system (reviewed in Kehrl, 1991; Ruscetti and Palladino, 1991). It inhibits the proliferation of thymocytes (Ristow, 1986), T cells, B cells (Kehrl *et al.*, 1986a,b), natural killer (NK) cells (Rook *et al.*, 1986), cytotoxic T cells (Ranges *et al.*, 1987), and lymphokine-activated killer (LAK) cells (Espevik *et al.*, 1988). The cytolytic activity of cytotoxic T cells (Ranges *et al.*, 1987) and NK cells (Rook *et al.*, 1986) and adhesion of T lymphocytes and neutrophils to the endothelium (Gamble and Vadas, 1988) are inhibited by TGF-β. The production and secretion of immunoglobulins (IgG and IgM) by B cells is impaired by TGF-β (Kehrl *et al.*, 1986a). In contrast, TGF-β induces B cell isotype switching to IgA in murine B cells stimulated with lipopolysaccharide (LPS) (Coffman *et al.*, 1989), although the IgA secretion is inhibited under the continued presence of TGF-β.

5. *Monocytes/Macrophages*

TGF-β induces chemotaxis of monocytes (Wahl *et al.*, 1987), but acts as a negative regulator on most macrophage functions, e.g., it suppresses hydrogen peroxide secretion (Tsunawaki *et al.*, 1988) and the interferon (IFN)-γ-induced expression of the major histocompatibility complex (MHC) class II molecules, which are important for the presentation of antigen for T cells (Czarniecki *et al.*, 1988).

6. *Cytokines and Their Receptors*

The production of cytokines is also regulated by TGF-β. For example, TGF-β induces an increase in the mRNA levels for IL-1 and tumor necrosis factor (TNF) α in monocytes, but suppress the LPS-induced synthesis of these mRNAs (Chantry *et al.*, 1989; Espevik *et al.*, 1987). The production of IFN-γ by mononuclear cells is inhibited by TGF-β (Espevik *et al.*, 1987). TGF-β has an autoinductive effect, and each isoform stimulates the production of itself and of the other isoforms (Van Obberghen-Schilling *et al.*, 1988; Bascom *et al.*, 1989). Certain cytokine receptors are down-regulated by TGF-β, including the receptors for GM-CSF, IL-3, and granulocyte colony-stimulating factor (G-CSF) in the murine myeloid cell lines (Jacobsen *et al.*, 1991), the IL-1 receptor in T and B lymphocytes (Dubois *et al.*, 1990), the IL-2 receptor α chain in large granular lymphocytes (Ortaldo *et al.*, 1991), and IFN-γ receptor in macrophages (Pinson *et al.*, 1992).

7. Immune Functions in Vivo

Systemic administration of TGF-β results in the suppression of immune functions, therefore TGF-β has been shown to have beneficial effects, e.g., on ectopic cardiac transplantation (Wallick *et al.*, 1990; Waltenberger *et al.*, 1993) and relapsing experimental allergic encephalomyelitis (Kuruvilla *et al.*, 1991; Johns *et al.*, 1991; Miller *et al.*, 1992).

The TGF-β1 gene was recently disrupted by homologous recombination (Shull *et al.*, 1992; Kulkarni *et al.*, 1993); mice that do not produce TGF-β1 were found not to have any major developmental abnormalities, but some intrauterine death was observed (Kulkarni *et al.*, 1993). About 20 days after birth, they died of an acute wasting syndrome with a multifocal, mixed inflammatory cell infiltration and tissue necrosis in several organs, such as heart, stomach, liver, lung, pancreas, salivary gland, and striated muscle. The infiltrates were mainly lymphocytes and neutrophils; the histopathology of these tissues was similar to that found in myocarditis, polymyositis, and Sjögren's syndrome (Shull *et al.*, 1992). This suggests that the immunosuppressive effects may be one of the most important roles for TGF-β1 *in vivo*, and this may not be compensated by other TGF-β isoforms.

III. Serine/Threonine Kinase Receptors

It is likely that receptors with serine/threonine kinase activity are of particular importance in the signal transduction of the members of the TGF-β superfamily. The first member of this family isolated in animal cells was encoded by *Caenorhabditis elegans daf-1*, a gene that controls dauer larva development (Georgi *et al.*, 1990). The ligand(s) for Daf-1 have not been identified. Type II receptors for activin and TGF-β were subsequently also found to belong to the family of serine/threonine kinase receptors.

A. cDNA Cloning of the Activin and TGF-β Type II Receptors

By expression cloning using ^{125}I-labeled activin A to screen transfected COS cells, Mathews and Vale (1991) managed to clone a cDNA for activin receptor type II from AtT20 mouse corticotropic cells. The structure of the activin type II receptor comprises a cysteine-rich extracellular domain, a single transmembrane domain, and a cytoplasmic domain containing a putative serine/threonine kinase (Fig. 2). Activin receptor II also binds activin B, albeit with slightly lower affinity than binding to activin A. Recently, based on the sequence similarity be-

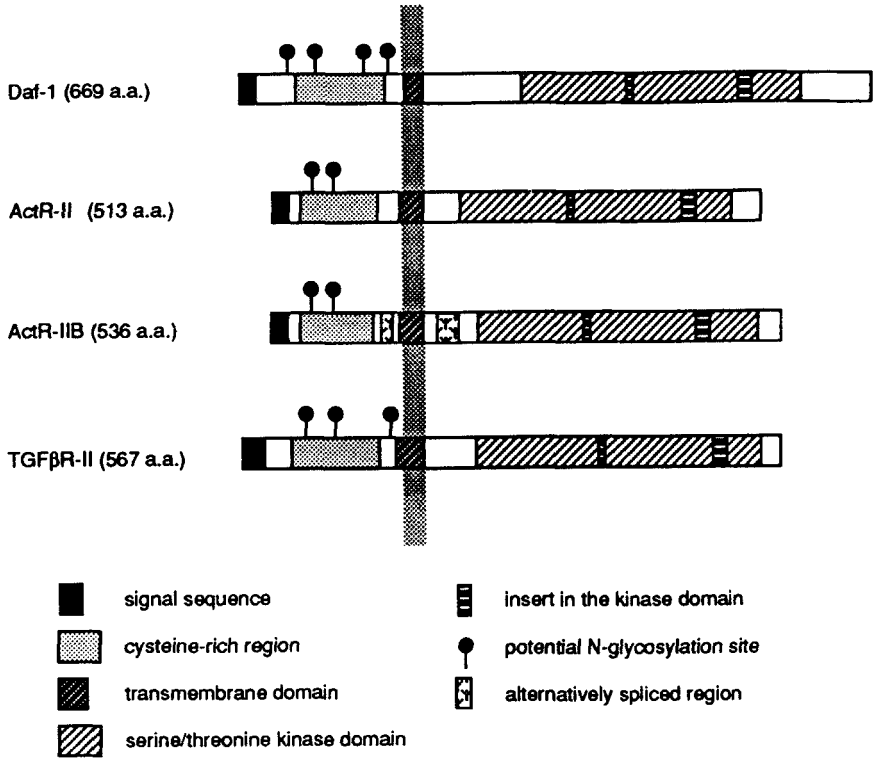
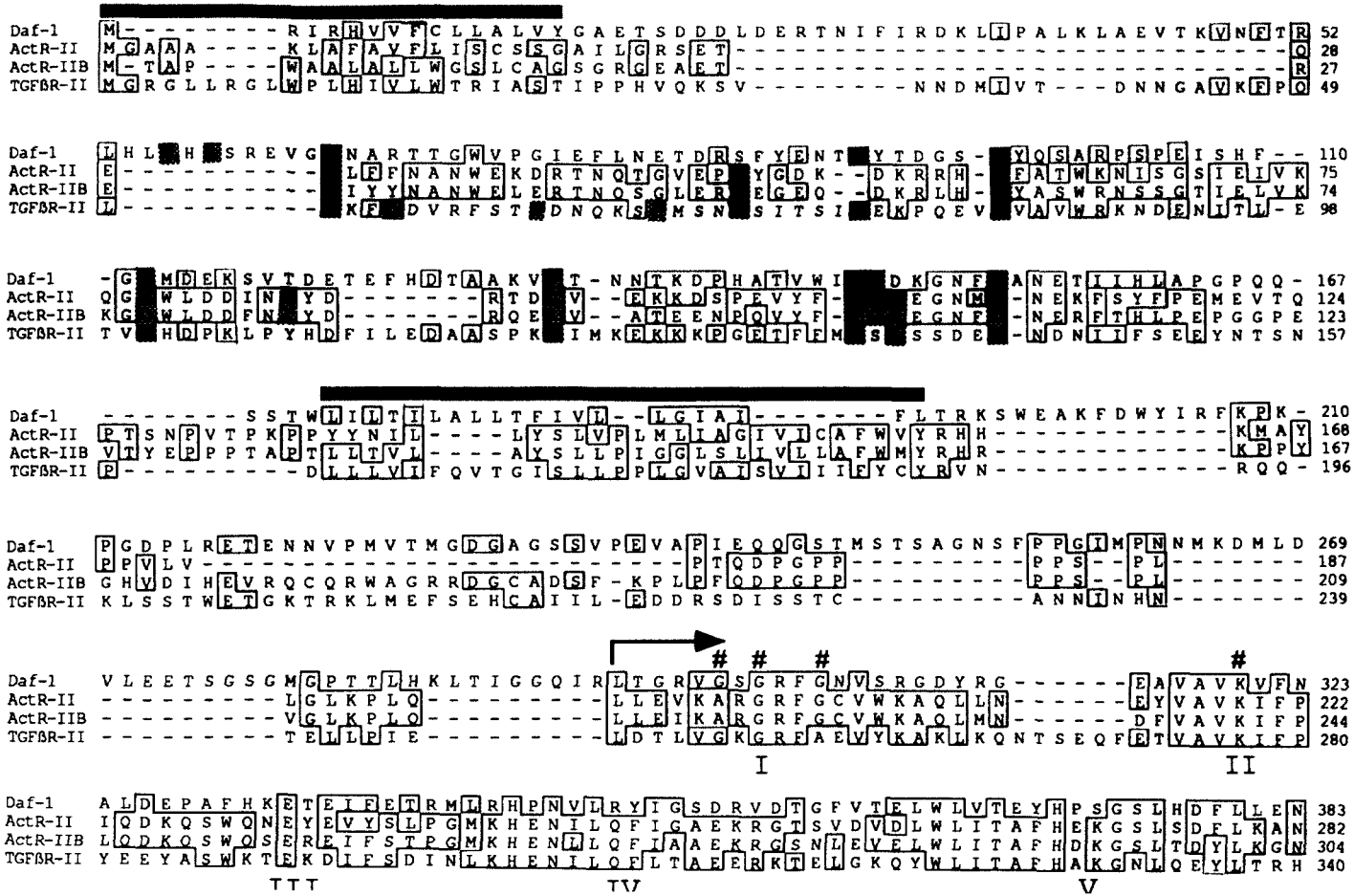


FIG. 2. Schematic illustration of the structures of Daf-1 (Georgi *et al.*, 1990), activin receptor II (ActR-II) (Mathews and Vale, 1991), activin receptor IIB (ActR-IIB) (Attisano *et al.*, 1992), and TGF-β type II receptor (TGFβR-II) (Lin *et al.*, 1992).

tween Daf-1 and activin receptor II, another activin type II receptor was identified, termed activin receptor type IIB (Attisano *et al.*, 1992; Mathews *et al.*, 1992; Legerski *et al.*, 1992; Nishimatsu *et al.*, 1992). Different spliced variants have been observed for mouse activin receptor IIB; an exon encoding a 24-amino acid segment rich in charged amino acid residues and another encoding an 8-amino acid segment rich in proline residues can be present or not in the cytoplasmic juxtamembrane domain and in the extracellular juxtamembrane domain, respectively (Fig. 2) (Attisano *et al.*, 1992). Among the four different isoforms of the activin receptor IIB that can be generated by the alternative splicing, those lacking the proline cluster in the extracellular part have a lower affinity for activin A than do those containing this segment. The existence of multiple activin receptor II isoforms may explain, in part, why different embryonic cell fates in *Xenopus* blasto-


Daf-1
ActR-II
ActR-IIB
TGFβR-II
I
II
III
IV
V

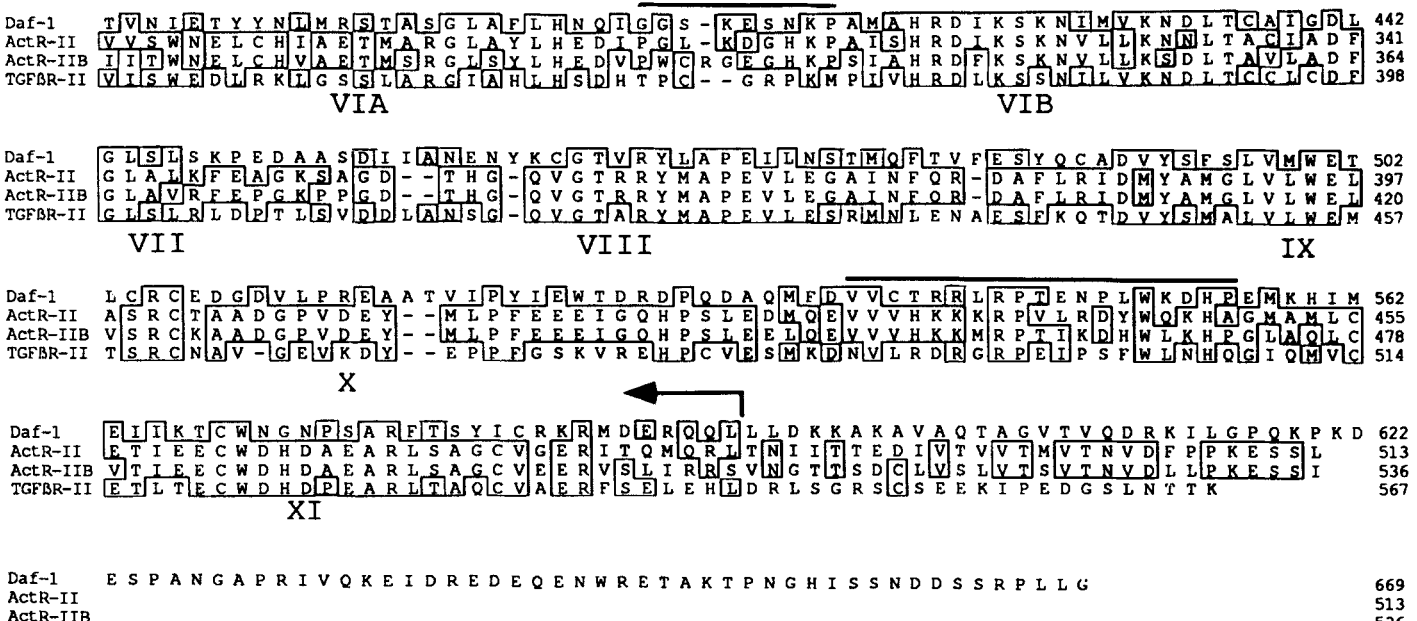


FIG. 3. Comparison of the amino acid sequences of *C. elegans* Daf-1, human activin receptor II (ActR-II), mouse activin receptor IIB (ActR-IIB), and human TGF-β receptor II (TGFβR-II). Sequences were obtained from GenBank (access numbers for Daf-1, ActR-II, ActR-IIB, and TGFβR-II are M32877, X63123, M84120, and M85079, respectively). Identical amino acid residues are boxed, cysteine residues in the extracellular domains are overlined by thick lines, inserts in the kinase domains are shown by thin lines, putative ATP-binding sequences in subdomains I and II are marked by the symbol #, and the borders of the kinase domains are indicated by arrows. Kinase subdomains are indicated with roman numerals according to the nomenclature of Hanks *et al.* (1988).

meres are obtained with very small differences (less than 2-fold) in activin concentrations (Green and Smith, 1990). Both activin receptors II and IIB show more than 10-fold lower affinity for inhibin A (Mathews and Vale, 1991; Attisano *et al.*, 1992). Thus, inhibins might possess their own specific receptor to elicit their biological effects.

The TGF-β type II receptor was identified as a glycoprotein with a molecular mass of 80 kDa by affinity cross-linking using radiolabeled TGF-β1 (Cheifetz *et al.*, 1988a). The TGF-β receptor II has a ubiquitous expression pattern and is often found coexpressed with the type I receptors (Massagué *et al.*, 1990), with which it may form a heteromeric complex (see Section V). Recently, the cDNA encoding the TGF-β receptor II was isolated by an expression cloning strategy (Lin *et al.*, 1992). Expression of the TGF-β receptor II cDNA in COS cells revealed an 80-kDa cell surface protein that specifically bound ^{125}I-labeled TGF-β1. The TGF-β receptor II was found to contain a cytoplasmic protein kinase domain with predicted serine/threonine kinase activity.

B. Structural Comparison of Type II Receptors for TGF-β and Activin

The domain structures of Daf-1, activin receptors II and IIB, and the TGF-β receptor II are similar; an N-terminal hydrophobic signal sequence is followed by a cysteine-rich ligand-binding domain, a single hydrophobic transmembrane region, and an intracellular portion, which consists almost entirely of a kinase domain (Figs. 2 and 3). Typical of most protein kinases, a catalytic domain containing 12 subdomains with conserved amino acid residues is found (Hanks *et al.*, 1988), suggesting that these receptors are functional protein kinases. A conserved sequence, Gly-X-Gly-X-X-Gly-X_{11-28}-Lys, which is thought to form part of the binding site of ATP, is present in Daf-1. In the TGF-β receptor II and activin receptors II and IIB, there are deviations from this consensus sequence (Fig. 3), which have also been described in other kinases (Hanks *et al.*, 1988).

Injection of mRNA for activin receptor II or IIB into *Xenopus* oocytes was shown to increase the responsiveness to activin and induce a secondary body axis formation (Kondo *et al.*, 1991; Mathews *et al.*, 1992); injection of mRNA encoding a truncated activin receptor II, lacking a kinase domain, was shown to function as a dominant negative receptor (Hemmati-Brivanlou and Melton, 1992), thus indicating that the protein kinase domain is essential for signal transduction by the type II receptors.

The kinase domains of the TGF-β receptor II, activin receptors II and IIB, and Daf-1 show about equal overall sequence similarity with tyrosine and serine/threonine kinases. Subdomains VI and VIII are known to contain amino acid sequences that allow prediction of the specificity of phosphorylation toward tyrosine versus serine/threonine residues (Hanks *et al.*, 1988). Analysis of the amino acid sequence of the TGF-β receptor II, activin receptors II and IIB, and Daf-1 in this region predict them to be serine/threonine kinases. In addition, these receptors do not have any tyrosine residues between subdomains VII and VIII, although tyrosine residues are commonly found at these regions in protein tyrosine kinases (Hanks *et al.*, 1988). Moreover, a bacterial fusion protein containing the intracellular domain of the human TGF-β receptor II was shown to autophosphorylate on serine and threonine residues (Lin *et al.*, 1992), thus showing that this family of receptors possesses a functional intrinsic serine/threonine kinase activity. However, purified mouse activin receptor IIB was shown to be a dual kinase, which autophosphorylates as well as phosphorylates exogenous substrates on serine, threonine, and tyrosine residues (Nakamura *et al.*, 1992).

A unique characteristic of the members of this receptor serine/threonine kinase family is the presence of two short kinase inserts between subdomains VIA and VIB and between subdomains X and XI (Fig. 3). In the intracellular domains, these regions, together with the juxtamembrane parts and the C-terminal tails, are most divergent between the members of the family. In receptor tyrosine kinases these regions contain tyrosine autophosphorylation sites, which provide attachment sites for substrates or regulate the kinase activity (Ullrich and Schlessinger, 1990). For the TGF-β receptor II, activin receptor II, and Daf-1, serine and threonine residues that could function as autophosphorylation sites are present in these regions, most abundantly in the C-terminal tails (Fig. 3).

The extracellular domains of the receptors show little sequence similarity, although cysteine-rich regions are found in all family members. The position of the cysteine residues can be aligned, suggesting that the extracellular domains may have a similar structural configuration. Activin receptors II and IIB are also highly conserved during evolution; there are only two amino acid residue changes in comparing the human and mouse activin receptor II; these changes are found in the extracellular domain (Donaldson *et al.*, 1992; Matzuk and Bradley, 1992). The extracellular domain of the TGF-β receptor II is less conserved, with 17 amino acid residue differences in human and mink receptors (Wrana *et al.*, 1992).

C. Type II Receptor Binds Three TGF-β Isoforms

It has been shown that the overall population of TGF-β receptors II and I preferably bind TGF-β1 and TGF-β3 (K_d of about 5 to 50 p*M*) compared to TGF-β2 (K_d of about 500 p*M*) in most cell types, including mink lung epithelial (Mv1Lu) cells and fetal bovine heart endothelial (FBHE) cells (Cheifetz *et al.*, 1990). This difference in binding affinity of the different isoforms corresponds well to the at least 50-fold higher biological potency of TGF-β1 and TGF-β3 versus TGF-β2 on FBHE cells. Mv1Lu cells, however, respond with equal potency to all three isoforms. A possible explanation for this finding has come through analysis of TGF-β receptors on Mv1Lu cells; in addition to a type II receptor population that binds TGF-β1 and TGF-β3 with high affinity and TGF-β2 with low affinity, these cells have a small subset of receptors that binds all three isoforms with high affinity (Cheifetz *et al.*, 1990). The mechanism by which the subtypes of the type II receptor are generated, remains to be elucidated. Wrana *et al.* (1992) showed that the cloned TGF-β receptor II gene encodes both high- and low-affinity receptors for TGF-β2 and elicits growth inhibitory activity with similar potencies by the three isoforms, which suggests that complex formation of type II receptors and other components may account for the different affinity classes of type II receptors (see Section V).

D. Other Serine/Threonine Kinase Receptors

The similarity in the structures of the extracellular domains in the TGF-β receptor II and activin receptors II and IIB suggests that other members of the TGF-β superfamily, including inhibins, BMPs, and MIS, may bind to receptors that belong to the same receptor family. Based on the short stretches of conserved amino acid residues among the members of this family, and using a polymerase chain reaction (PCR)-aided approach, five novel structurally related receptors, termed activin receptor-like kinases (ALKs), have been identified (ten Dijke *et al.*, 1993). The ligands for ALKs have not yet been identified. Since ALKs have a higher sequence similarity toward each other than to other serine/threonine kinases, they may bind related ligand(s), possibly belonging to the TGF-β superfamily. The expression pattern for each ALK is distinct, indicating different roles for each ALK protein *in vivo*.

The type V TGF-β receptor was recently purified from bovine liver membrane as a 400-kDa component (O'Grady *et al.*, 1991a), and has been found to be expressed widely in various cell types (O'Grady *et al.*, 1991b). TGF-β1 binds to the type V receptor, and the binding is

completely competed for by TGF-β1 and TGF-β2 (O'Grady *et al.*, 1991b). Recently, the TGF-β type V receptor was shown to be a functional serine/threonine kinase (O'Grady *et al.*, 1992). Since this receptor has not been cloned, its structural similarity to the TGF-β receptor II is not known. However, limited amino acid sequence analysis revealed that the ATP-binding region has a high degree of similarity to members of the serine/threonine kinase receptor family. Moreover, TGF-β stimulated the serine/threonine-specific autophosphorylation of the TGF-β type V receptor as well as the phosphorylation of an exogenous substrate, casein, suggesting that receptor V is important for TGF-β signaling.

IV. Other TGF-β Receptors

In addition to the serine/threonine kinase types of TGF-β receptors (type II and V), about 10 different types of cell surface TGF-β receptors or binding proteins have been identified in various cells and tissues by affinity cross-linking with radioiodinated TGF-βs. These include type I, type III, type IV, and type VI TGF-β receptors, endoglin, and several other binding proteins with different molecular sizes and characteristics. Among these cell surface TGF-β receptors, the type I and type III receptors were initially identified in many cell lines that respond to TGF-β, and thus have been most extensively investigated.

A. TGF-β Type I Receptor

Studies on chemically induced TGF-β receptor mutants (Laiho *et al.*, 1991a; Wrana *et al.*, 1992) have indicated that both type I and type II receptors are indispensable for the transduction of the growth inhibitory signal of TGF-β (see Section V).

Cross-linked components of about 65 kDa corresponding to the type I receptor have also been found for activin A (Hino *et al.*, 1989; Mathews and Vale, 1991) and BMP-4 (Paralkar *et al.*, 1991a). However, the structures of the type I receptors for members of the TGF-β superfamily have not been elucidated in detail. It appears unlikely that TGF-β receptor I is a truncated TGF-β receptor II, because of their different protease digestion patterns, different sensitivity to reducing agents, and distinct binding affinities for different TGF-β isoforms (Cheifetz *et al.*, 1990; Cheifetz and Massagué, 1991). The possibility remains open that the TGF-β type I receptor has its own intrinsic kinase domain; if so, considering its molecular mass of 47 kDa after removal of carbohydrates (Cheifetz *et al.*, 1988a), and the fact that about 280 amino acid residues are needed to constitute a catalytic

kinase domain (Hanks *et al.*, 1988), the type I receptor would have a very short ligand-binding domain.

Certain hematopoietic cells (Ohta *et al.*, 1987; Brandes *et al.*, 1991a,b) and transformed cells (Geiser *et al.*, 1992; Ichijo *et al.*, 1990) express predominantly the type I receptor. Whether these cells express a subtype of receptor II, for which cross-linking occurs less efficiently, or receptor I mediates signals in the presence of only a low number of the type II receptor (see Section V), remain to be determined.

B. TGF-β Type III Receptor (Betaglycan) and Endoglin

TGF-β type III receptor is a transmembrane proteoglycan composed of a 120-kDa core protein and large glycosaminoglycan (GAG) side chains that are rich in heparan sulfate and chondroitin sulfate (Segarini and Seyedin, 1988). Therefore, the TGF-β type III receptor is also called betaglycan (Andres *et al.*, 1989). Cloning of the rat TGF-β type III receptor gene (López-Casillas *et al.*, 1991; Wang *et al.*, 1991) revealed that the rat receptor III protein is composed of 853 amino acids consisting of an N-terminal large extracellular domain, a single transmembrane domain, and a short C-terminal intracellular domain of 43 amino acid residues (Fig. 4). The overall amino acid sequence identities of the human receptor compared with those of porcine and rat cells are ~80% (Morén *et al.*, 1992), which is relatively low compared to the high sequence conservation of their ligands (Derynck *et al.*, 1986; Miller *et al.*, 1989a,b). Two potential GAG attachment sites were conserved in all three species (Ser^{532}-Gly and Ser^{543}-Gly in human) as well as a putative dibasic proteolytic cleavage site (Lys^{743}-Lys), which may generate a soluble form of the type III receptor such as those found in the conditioned media of various cell types (Andres *et al.*, 1989). The short intracellular domain of the type III receptor does not appear to contain any enzymatic activity.

Although many cell lines express receptor III as well as receptors I and II, certain TGF-β-responsive cells, e.g., hematopoietic progenitor cells (Ohta *et al.*, 1987), endothelial cells (Cheifetz *et al.*, 1990), and primary (or early passage) cultured epithelial cells (Segarini *et al.*, 1989), were found not to express receptor III. These observations show that the type III receptor is not necessary for TGF-β signaling.

Receptor III binds TGF-β1, TGF-β2, as well as TGF-β3 with K_d values of 50 to 200 p*M*, which are about 10-fold higher than the K_d values of receptors I and II for TGF-β1 and TGF-β3. Subtypes of TGF-β receptor III with different affinities for TGF-β isoforms have

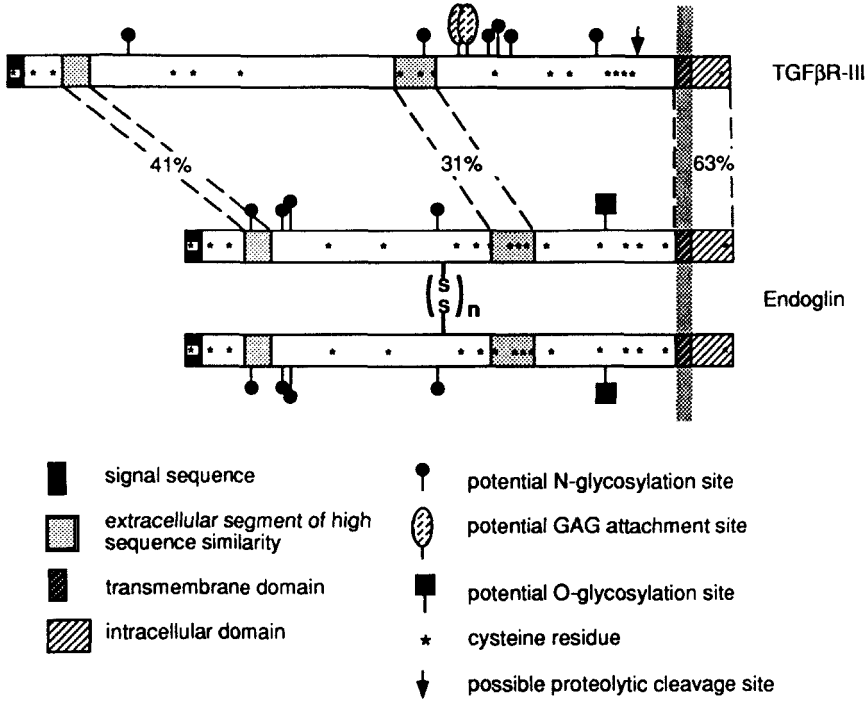


FIG. 4. Schematic illustration of the TGF-β type III receptor (TGFβR-III) and endoglin. The percentages of identical amino acids in specific regions of the human TGF-β type III receptor (Morèn *et al.*, 1992) and human endoglin (Gougos and Letarte, 1990) are shown. Note that the positions of the O-glycosylation site(s) and interchain disulfide bridge(s) in endoglin have not been precisely localized.

been demonstrated in certain cell types (Segarini *et al.*, 1987; Mitchell and O'Connor-McCourt, 1991; Mitchell *et al.*, 1992a); the relation between these molecules and the cloned type III receptor remains to be determined.

In addition to binding TGF-β with its core protein part (Cheifetz and Massagué, 1989), TGF-β receptor III has been shown to bind basic fibroblast growth factor (FGF) with its carbohydrate chains (Andres *et al.*, 1992). Basic FGF needs to bind heparin or heparan sulfate in order to elicit the biological responses via its signaling tyrosine kinase receptors. Therefore, it is possible that TGF-β receptor III modulates the activity of two different growth regulatory factors.

TGF-β receptor III has been shown to have significant sequence

similarity to several membrane protein sequences, including those of sperm receptors Zp2 and Zp3, urinary protein uromodulin, major zymogen granule membrane glycoprotein GP-2 (Bork and Sander, 1992), and endoglin (Gougos and Letarte, 1990). The similarity between TGF-β receptor III and endoglin is strikingly high (about 70%) in the transmembrane and intracellular regions.

Endoglin is a major glycoprotein of human vascular endothelium, and has a homodimeric structure (180 kDa) of disulfide-linked monomers of 95 kDa (Gougos and Letarte, 1988b). Endoglin was originally identified by a monoclonal antibody generated against the pre-B leukemic cell line HOON. Analysis by immunohistochemistry revealed that it is highly expressed in endothelial cells and in the mesangium of the kidney (Quackenbush *et al.*, 1986; Gougos and Letarte, 1988a). cDNA cloning of human endoglin revealed that it is composed of an extracellular domain of 561 amino acid residues, a single transmembrane domain, and a short intracellular domain of 47 amino acid residues (Fig. 4) (Gougos and Letarte, 1990). In addition to the transmembrane and intracellular domains, two portions of 29 and 52 amino acid residues, which were substantially similar to endoglin, were found in the extracellular domain of TGF-β receptor III (Morén *et al.*, 1992). Moreover, endoglin has recently been shown to bind TGF-β in an isoform-specific manner, i.e., TGF-β1 (K_d of about 50 p*M*) and TGF-β3 (but not TGF-β2) bind endoglin (Cheifetz *et al.*, 1992). This is in contrast to the TGF-β type III receptor, which binds all three TGF-β isoforms (López-Casillas *et al.*, 1991). The human endoglin has an Arg–Gly–Asp (RGD) tripeptide, a possible binding site to cell adhesion receptors (Gougos and Letarte, 1990). However, the RGD tripeptide is not conserved in porcine endoglin (K. Miyazono, unpublished observation), thus rendering it unlikely that the RGD motif is important for the function of endoglin.

C. TGF-β Type IV Receptor

The type IV TGF-β receptor was identified as a 70- to 74-kDa cross-linked complex with the TGF-β monomer, i.e., a molecular size similar to that of the type I receptor. However, the type IV receptor is unique in that it has so far been found only in GH_3 pituitary tumor cells and in that it binds not only TGF-β, but also activin and inhibin (Cheifetz *et al.*, 1988b). Because activin and inhibin are known to modulate the production and release of FSH in cultured pituitary cells, and no other types of TGF-β receptors were detected in the GH_3 cell line, it is possible that the biological effects of TGF-β, activin, and inhibin on this particular cell line may be elicited through the type IV receptor.

D. TGF-β Type VI Receptor and Isoform-Specific TGF-β-Binding Proteins

Type VI receptor, found in L6 myoblasts and A549 lung adenocarcinoma cells as a glycoprotein of 180 kDa, shows binding to TGF-β1 only in the presence of TGF-β2 (Segarini *et al.*, 1992). Certain other TGF-β-binding proteins, which bind TGF-β1 but not TGF-β2 and have molecular sizes similar to that of the type VI receptor, have been reported. These include 150- and 180-kDa proteins in several different cells (MacKay and Danielpour, 1991), and a 180-kDa phosphatidylinositol (PI)-anchored membrane protein in FBHE cells and MG-63 human osteosarcoma cells (Cheifetz and Massagué, 1991). Despite the fact that the type VI receptor binds TGF-β1 only in the presence of TGF-β2, it is possible that some of these molecules are identical to the type VI receptor. In addition to the PI-anchored 180-kDa TGF-β1-binding protein, two other types of PI-anchored proteins, of 60 and 140 kDa, specifically bind TGF-β2 (Cheifetz and Massagué, 1991). A 38-kDa TGF-β-binding glycoprotein with 5- to 10-fold higher affinity for TGF-β2 than TGF-β1 was observed in the choriocarcinoma cell line BeWo (Mitchell *et al.*, 1992b). The functions of these isoform-specific TGF-β-binding proteins are unknown; possible roles in the selective presentation of specific TGF-β isoforms to signaling receptor(s) should be considered.

E. Soluble TGF-β-Binding Proteins

There exist a wide variety of soluble forms or ECM forms of TGF-β-binding proteins, including β-LAPs (see Section I), a soluble form of the type III receptor (Andres *et al.*, 1989), α_2-macroglobulin (O'Connor-McCourt and Wakefield, 1987; Huang *et al.*, 1988), thrombospondin (Murphy-Ullrich *et al.*, 1992), β-amyloid precursor protein (Bodmer *et al.*, 1990), decorin, biglycan (Yamaguchi *et al.*, 1990), type IV collagen (Paralkar *et al.*, 1991b), α-fetoprotein (Altman *et al.*, 1990), and fibronectin (Fava and McClure, 1987). In addition, TGF-β-binding proteins of 40, 80, and 160 kDa have been identified and purified from porcine uterus membranes (Ichijo *et al.*, 1991). Recent cDNA cloning of the 40-kDa molecule has revealed that this molecule is a secreted protein with a structure containing fibrinogen- and collagen-like domains, therefore the protein was denoted ficolin. The 80- and 160-kDa molecules are multimers of the 40-kDa protein (Ichijo *et al.*, 1993). The recombinant ficolin did not show TGF-β-binding activity; it is possible that structural differences prevail between natural and recombinant forms of ficolin, which may affect the ability to bind TGF-β.

Binding of α_2-macroglobulin and decorin results in the inactivation of TGF-β activity (O'Connor-McCourt and Wakefield, 1987; Huang *et al.*, 1988; Yamaguchi *et al.*, 1990), whereas inhibition of TGF-β activity by other TGF-β-binding proteins has not been observed. Decorin, despite its relatively low affinity to TGF-β, has been reported to prevent the development of glomerulonephritis caused by TGF-β (Border *et al.*, 1992). Thus, TGF-β-binding proteins may act as scavengers of excess TGF-β and restrict the action of TGF-β; moreover, they may form reservoirs for TGF-β and keep TGF-β available for immediate use when needed.

V. Signal Transduction by TGF-β

A. Formation of TGF-β Receptor Complex(es) for Intracellular Signaling

Studies on chemically mutagenized mink lung epithelial cells, Mv1Lu, which are resistant to growth inhibition by TGF-β, have suggested that both receptors I and II are directly involved in signal transduction and interact with each other in a receptor complex. Two types of TGF-β receptor mutant phenotypes were isolated: mutants that by cross-linking showed no binding to receptor I, but showed normal binding to receptor II (class R), and mutants that were defective in binding or showed anomalous binding to both receptors I and II (class DR) (Boyd and Massagué, 1989; Laiho *et al.*, 1990a). No mutants were isolated that lacked only binding to receptor II. Somatic cell hybrids between class R and DR mutant phenotypes rescue receptor I (Laiho *et al.*, 1991a), suggesting that receptor I is present in the DR mutants. These data suggest that receptor II alone can bind TGF-β, but receptor I may need receptor II to bind TGF-β, or possibly receptor I depends on receptor II to reach to the cell surface (Lin and Lodish, 1993). All mutants selected for resistance to TGF-β growth inhibition were also defective in other TGF-β responses, including lack of up-regulation of early response genes, as well as change in cell morphology on addition of TGF-β. This suggests that receptors I and II are involved in many of the responses mediated by TGF-β. Additional evidence for cooperativity between receptors I and II was obtained by showing that receptors I and II can be coimmunoprecipitated, that TGF-β receptor II transfected into DR mutants restored receptor I binding and made cells responsive to TGF-β, and that a kinase-defective TGF-β receptor II mutant transfected into DR mutants was able to restore binding to receptor I, but was not able to mediate

TGF-β signaling (Wrana *et al.*, 1992). Thus, receptor I needs receptor II to bind TGF-β and receptor II needs receptor I to transduce signals (Fig. 5A). Receptor I appears to be acting downstream of receptor II, possibly regulating the kinase activity of receptor II. Receptor I may provide receptor II with the appropriate substrates or may be a substrate for receptor II.

The first step in the activation of many tyrosine kinase receptors is a ligand-induced dimerization (Ullrich and Schlessinger, 1990). This induces a trans autophosphorylation between the receptors in the dimer. It remains to be elucidated whether the dimeric TGF-β molecule induces dimerization of TGF-β receptor II, with concomitant activation of its cytoplasmic protein serine/threonine kinase activity, autophosphorylation, and association with and activation of cellular substrates. It also remains to be determined whether the complex between TGF-β receptors I and II is a heterodimeric or a heterotetrameric complex (Fig. 5A).

A bladder carcinoma cell EJ and a colon adenocarcinoma cell SW480 predominantly express receptor I (and receptor III), but less receptor II. These cells do not respond to TGF-β by growth inhibition, but respond by synthesis of fibronectin, type IV collagenase, and PAI-1 (Geiser *et al.*, 1992). Fusion of EJ and SW480 cells results in the appearance of receptor II on the cell surface and concomitantly cells acquire the ability to respond to TGF-β by growth inhibition. It is an interesting possibility, which remains to be elucidated, that receptor I of EJ and SW480 cells can bind TGF-β in the presence of low numbers of receptor II or other components, and that receptor I may be responsible for transducing the signals leading to the production of fibronectin, type IV collagenase, and PAI-1 (Geiser *et al.*, 1992), whereas the growth inhibitory effect of TGF-β is more dependent on the type II receptor.

Cancer cells often acquire resistance to TGF-β and thus escape from the negative growth control exerted by TGF-β (reviewed in Roberts and Sporn, 1990). Alteration of the expression of the TGF-β receptors by cellular transformation has been shown in several different cells, e.g., loss of type I, II, and III receptors was shown in certain retinoblastoma cells (Kimchi *et al.*, 1988). Moreover, overexpression of the H-*ras* oncogene in the epithelial cell line IEC-18 resulted in decreased binding of TGF-β to the type II receptor and resistance to TGF-β growth inhibition (Filmus *et al.*, 1992). Wrana *et al.* (1992) observed that certain of the class DR mutants of Mv1Lu cells showed point mutations in the extracellular or transmembrane domain of the type II receptor. Thus, resistance to TGF-β action may involve alterations in

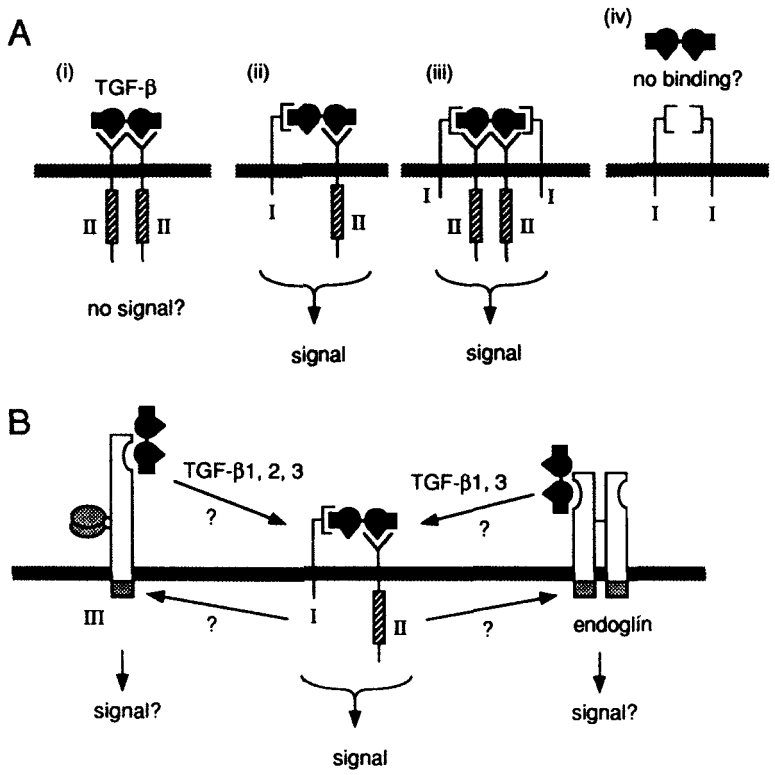


FIG. 5. Schematic illustration of possible models of signaling through heteromeric TGF-β receptor complexes. (A) Signaling through heteromeric receptor I and II complexes. The type II receptor alone cannot transduce signal efficiently (i). Heteromers of one molecule each (ii) or two molecules each (iii) of TGF-β receptors I and II give efficient signal transduction; available data do not make it possible to distinguish between the heterodimeric and heterotetrameric models for signal transduction. Type I receptor alone cannot bind TGF-β with high affinity (iv); the alternative possibility that receptor I is dependent on receptor II to reach the cell surface has not been ruled out. (B) Role of receptor III and endoglin in the interaction with signaling receptors. The type III receptor binds three TGF-β isoforms, but endoglin binds only TGF-β1 and TGF-β3. These receptors may present ligands to signaling receptors I and II. They may also be phosphorylatd by the signaling receptors and be involved in the transduction of signals, e.g., by providing attachment sites for downstream components in the signal transduction pathway. For further discussion and references, see the text.

receptor II, or other TGF-β receptors or downstream components in the signal transduction pathway. An important aim for future studies will be to explore if perturbations of the TGF-β signaling pathway are important for cellular transformation.

B. Possible Functions of Type III Receptor and Endoglin

What is the role of TGF-β receptor III in signaling TGF-β? Whereas receptors I and II are present on most cells that respond to TGF-β, receptor III is not detectable in certain cell types (Massagué *et al.*, 1990). Furthermore, none of the mutagenized Mv1Lu cells that are resistant to TGF-β is affected in receptor III binding (Laiho *et al.*, 1990a). TGF-β receptor III, as well as endoglin, have short intracellular domains with no obvious signaling motif, but are very rich in serine and threonine residues (42%) (see Fig. 4). An interesting possibility, which remains to be elucidated, is that the intracellular domain of type III receptors might be phosphorylated in a heteromeric complex by the type II and type I receptors, and thereby is involved in signal transduction (Fig. 5B). Another possibility is that receptor III has a more indirect function; i.e., it may act as a reservoir and/or a presentor of TGF-β for the signaling receptors (Fig. 5B). The facts that the type III receptor is more abundantly expressed and that its affinity for TGF-β is lower than that of receptors I and II are compatible with such a role. Stable expression of receptor III into L6 myoblasts increased the TGF-β binding to receptor II (Wang *et al.*, 1991). This effect was also observed by the transfection of a C-terminally truncated receptor III that lacked most of the intracellular portion (Wang *et al.*, 1991); thus, the facilitated binding of TGF-β to receptor II appears to be mediated solely by binding of TGF-β to the extracellular part of receptor III. The mechanism for the interaction between receptors III and II remains to be elucidated.

Whereas the TGF-β type III receptor shows about equal affinity for TGF-β1, TGF-β2, and TGF-β3, endoglin shows high affinity binding to TGF-β1 and TGF-β3, but not to TGF-β2. Importantly, it has been reported that endothelial cells, which lack the type III receptor, respond well to TGF-β1 and TGF-β3 but not to TGF-β2 (Jennings *et al.*, 1988; Cheifetz *et al.*, 1990; Hirai and Kaji, 1992). The correlation between the binding of the different TGF-β isoforms to endoglin and their activity on endothelial cells suggest that endoglin may regulate the TGF-β activity by means of isoform-specific ligand presentation to signaling receptors (Fig. 5B). Alternatively, similar to the possible role of receptor III (discussed above), endoglin could act as an accessory molecule for the signal transduction of TGF-β, cooperating with receptors I and II. MacKay and colleagues (1990, 1992) reported TGF-β1-specific binding proteins in rat glomeruli. These proteins may be related to endoglin and are disulfide-linked multimers of 320, 260, 170, and 85 kDa. Interestingly, the 260-kDa cross-linked complex was composed of an endoglin-like 170-kDa protein and a TGF-β receptor II-

like 85-kDa protein (MacKay *et al.*, 1992). These results suggest that endoglin may physically interact with the TGF-β receptor II. Endoglin and receptor III have a structural similarity and thus they may have similar functions.

C. Intracellular Signals Induced by TGF-β

The intracellular signaling pathways available after activation of the TGF-β receptors are still not well understood; substrates for the receptor kinase remain to be identified and the mechanism for signaling through the cytoplasm is virtually unknown. However, some information is available concerning nuclear events in TGF-β-stimulated cells (reviewed in Moses *et al.*, 1990); recent studies revealed the involvement of the retinoblastoma tumor suppressor gene product RB in the growth inhibitory signal of TGF-β. TGF-β inhibits the growth of cells by interrupting the cell cycle at middle to late G_1 phase (Laiho *et al.*, 1990b). In Mv1Lu cells and human monocytic leukemia cells, growth inhibition induced by TGF-β was shown to be associated with the inhibition of RB phosphorylation (Laiho *et al.*, 1990b; Furukawa *et al.*, 1992). Moreover, $p34^{cdc2}$ protein kinase activity was shown to be inhibited by TGF-β (Howe *et al.*, 1991; Furukawa *et al.*, 1992; Abraham *et al.*, 1992). Thus, suppression of RB phosphorylation may be regulated through inhibition of members of the cdc2 kinase family by TGF-β. However, loss of the RB protein by deletion or rearrangement of both *RB1* gene copies did not affect the response to TGF-β in breast cancer cells, suggesting the presence of additional mechanisms for growth inhibition by TGF-β (Ong *et al.*, 1991).

Repression of c-*myc* may also be an important signal for the growth inhibitory activity of TGF-β. Keratinocytes that were growth inhibited by TGF-β showed a decreased expression of c-*myc* (Coffey *et al.*, 1988; Pietenpol *et al.*, 1990a); the pathway leading to repression of c-*myc* may involve RB as well as other cellular proteins (Pietenpol *et al.*, 1990b, 1991). The down-regulation of c-*myc* is, however, not restricted to the G_1 phase, which suggests the presence of a cell cycle-independent TGF-β-regulated pathway leading to c-*myc* repression (Zentella *et al.*, 1991; Münger *et al.*, 1992).

The growth stimulation by TGF-β observed in certain mesenchymal cells may be induced by an indirect mechanism involving platelet-derived growth factor (PDGF) (Leof *et al.*, 1986). Growth stimulation of the AKR-2B cells occurred after a prolonged prereplicative phase (more than 24 hours) (Shipley *et al.*, 1985), which may be due to the induction of PDGF B chain and autocrine activation of a PDGF-

dependent growth stimulatory pathway (Leof *et al.*, 1986). Production of PDGF A chain is also induced in human smooth muscle cells and fibroblasts by low concentrations of TGF-β (Battegay *et al.*, 1990; Soma and Grotendorst, 1989), and the growth-promoting effect of TGF-β on these cells was abolished by anti-PDGF antibody (Battegay *et al.*, 1990). At high concentrations of TGF-β, the PDGF α receptor expression is decreased, which together with other growth inhibitory signals may dominate and result in growth inhibition (Battegay *et al.*, 1990).

The effects of TGF-β on ECM proteins appear to be mediated by transcriptional regulation of genes involved in ECM turnover. Transcriptional activation of the type I collagen and PAI-1 genes by TGF-β is mediated by specific TGF-β-responsive elements (Rossi *et al.*, 1988; Ritzenthaler *et al.*, 1991; Westerhausen *et al.*, 1991; Riccio *et al.*, 1992). Repression of the gene for transin/stromelysin, a matrix-degrading metalloproteinase, is regulated by the TGF-β1 inhibitory element (TIE) in the promoter, and is mediated by the binding of a protein complex containing Fos (Kerr *et al.*, 1990). Production of cytokines is also shown to be mediated by transcriptional regulation of genes; e.g., autoinduction of TGF-β1 is mediated by the AP-1 complex (Kim *et al.*, 1990), and inhibition of the IL-2 gene in T lymphocyets occurs through a noncanonical octamer-binding site in the IL-2 promoter region (Brabletz *et al.*, 1993).

TGF-β has also been shown to induce various other intracellular signals; however, it is currently not clear how these signals are involved in the signal transduction pathways of TGF-β. These include activation of $p21^{ras}$ (Mulder and Morris, 1992), inhibition of phospholipase C-induced hydrolysis of phosphatidylcholine (Diaz-Meco *et al.*, 1992), inhibition of the p53 tumor suppressor gene activity (Suzuki *et al.*, 1992; Landesman *et al.*, 1992), and activation of protein phosphatases (Gruppuso *et al.*, 1991). TGF-β induces a rapid phosphorylation of several nuclear proteins, including the transcription factor cyclic AMP-responsive element binding protein (CREB) (Kramer *et al.*, 1991). Induction of mRNA for some early response genes, e.g., *junB*, c-*fos*, and *JE*, has been observed in various cells (Pertovaara *et al.*, 1989; Laiho *et al.*, 1991b; Hanazawa *et al.*, 1991).

VI. Perspectives

Studies have revealed that TGF-β exerts its cellular effects by binding to a system of receptors that are quite different from tyrosine kinase receptors or cytokine receptors. The identification of the cDNA for TGF-β receptor II will allow further insights into the molecular mech-

anism of TGF-β action. A key element in the signal transduction is the intracellular serine/threonine kinase domain in the type II receptor. It will be important to determine whether the type II receptor undergoes autophosphorylation after ligand binding and, if so, to map the phosphorylation sites and determine whether they provide attachment sites for downstream components in the signal transduction pathway. Moreover, it will be important to identify substrates for the receptor kinase and determine the effects of phosphorylation on their activities.

The structure of the TGF-β type I receptor has yet to be identified. Cloning of its cDNA and further characterization will be necessary for a more complete understanding of TGF-β signaling and, in particular, of the mechanism for the interdependence of type I and type II receptors. An interesting question is whether there is a functional subspecialization between the two receptors so that they each mediate only part of the many effects of TGF-β on the responder cells. Progress in the characterization of the signaling receptors for TGF-β will also make it possible to determine at which level the growth inhibitory signals interfere with the growth stimulatory pathways originating from protein tyrosine kinase receptors and cytokine receptors.

A striking feature of TGF-β is its ability to interact with many different proteins, including signaling receptors, nonsignaling receptors, and soluble carrier proteins involved in delivery as well as scavenging of TGF-β. Recent elucidation of the 3-D structure of TGF-β2 will facilitate the understanding of the modes of interaction between TGF-βs and their receptors and binding proteins, and such information will be useful for the development of antagonists for TGF-β. The multitude of interactions suggests that the activity of TGF-β needs to be carefully controlled and modulated *in vivo*. Further knowledge about the regulation of TGF-β and its mechanism of action will help in understanding the pathogenesis of clinical disorders in which TGF-β is implicated, and to design treatment regimes for such conditions.

NOTE ADDED IN PROOF. Recent reports indicate that members of the ALK family of serine/threonine kinases can act as type I receptors for TGF-β or activin. ALK-1 [called TSR-I (Attisano *et al.*, 1993)] binds TGF-β or activin, ALK-2 [called Tsk 7L (Ebner *et al.*, 1993) or ActR-I (Attisano *et al.*, 1993)] binds TGF-β or activin and transduces an activin signal, and ALK-5 (Franzén *et al.*, 1993) binds TGF-β and transduces a TGF-β signal.

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Biochemistry of B Lymphocyte Activation

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I. Introduction

The primary function of B lymphocytes is to make antibodies against components of invading microorganisms. These antibodies facilitate the removal and destruction of pathogenic agents by activating the complement cascade and by serving as tags for endocytosis, phagocytosis, or antibody-dependent cellular cytotoxicity by macrophages, neutrophils, natural killer cells, or eosinophils. Antibodies can act locally at the site of an infection or they can be carried by the blood and the lymph to other parts of the body to fight microorganisms that have spread to other locations. Two antibody isotypes, IgA and IgM, can be transported into body secretions to fight invading organisms in these areas as well.

The process by which resting B cells are induced to proliferate and differentiate into antibody-secreting plasma cells is a complex, multistep process. B cell activation initially involves antigen binding to membrane forms of antibody molecules. Subsequent events vary depending on the nature of the antigen, but can include signals delivered by helper T cells or by macrophages. Receptors on B cells for antigen and for these additional regulatory molecules must therefore transduce signals across the plasma membrane. These signals must be integrated in some way and then translated into the biological responses that we observe, namely entry into the cell cycle, proliferation, and differentiation to antibody secretion. It has also become evident that cell survival is an important regulatory step in B cell activation. In this review we describe the current state of knowledge about the nature of these receptors, their signaling mechanisms, and the intracellular pathways that connect these events to longer term responses such as changes in gene expression. Although a considerable amount has been learned about the initial events that occur minutes to hours after receptor binding, there are still many important gaps in our knowledge. These gaps increase with increasing distance from the initial receptor-

binding events. Thus, very little is known about downstream intracellular events that result in the biological responses that often occur more than 24 hours after initial stimulation.

II. Different Models of B Cell Activation

The process of going from a resting B cell to an antibody-secreting cell can be divided into three stages: early activation, proliferation, and differentiation. The early activation of resting B cells is characterized by (1) exit from the resting state and entry into G_1 phase of the cell cycle; (2) increased synthesis of biosynthetic/secretory machinery such as ribosomes and endoplasmic reticulum (ER), which prepares the cell for high rate antibody secretion; (3) increased expression of class II major histocompatability (MHC) molecules, which facilitates antigen presentation to helper T cells; and (4) increased expression of receptors for cytokines made by activated helper T cells that drive B cell proliferation and antibody secretion.

The proliferative phase that follows early activation is responsible for greatly expanding the number of cells capable of producing antibodies against the stimulating antigen. This clonal expansion is followed by differentiation into plasma cells that secrete IgM. For some types of antigens (T dependent; see later) additional differentiative events occur. These include affinity maturation, the induction of memory B cells, and isotype switching.

B cell early activation can proceed by at least two fundamentally different mechanisms, one that requires contact with helper T cells and one that is stimulated by antigen contact in the absence of helper T cells. Thus, antigens can be classified as being T dependent or T independent.

The T-independent antigens can be divided into two types. Type 1 T-independent antigens generally contain molecules that activate B cells regardless of their antigen specificity; these molecules are called "polyclonal B cell activators." At high concentrations, polyclonal B cell activators will stimulate proliferation and antibody secretion by as many as one-third of all B cells (Andersson *et al.*, 1977). At low concentrations of the polyclonal B cell activator, however, much of the antibody made is directed against epitopes linked to the polyclonal B cell activator. It is under these conditions that polyclonal B cell activators can be considered antigens. These antigens can stimulate antibody production in animals lacking functional helper T cells and are thus truly T cell-independent antigens. Many polyclonal activators are

components of bacterial cell surfaces. The best studied of these is lipopolysaccharide (LPS), a major component of the outer membrane of gram-negative bacteria. LPS also activates macrophages, inducing them to secrete a large number of mediators, including the interleukin cytokines IL-1 and IL-6, which contribute to the generation of antibody-secreting cells (Kishimoto and Hirano, 1988). Corbel and Melchers (1983) have shown that macrophage-derived cytokines play an essential role in antibody responses to LPS. The mechanism by which polyclonal activators activate B cells is not understood, but it is thought that B cells express specific receptors for the stimulatory components of these types of antigens.

For antigens that are not polyclonal B cell activators, T-independent and T-dependent antigens represent a continuous spectrum from multivalent antigens that cross-link the B cell antigen receptor (AgR) extensively to monovalent antigens that cause little or no AgR cross-linking. Highly repetitious antigens such as repeating polysaccharide structures, or protein polymers such as bacterial flagellin, can stimulate B cell proliferation and IgM secretion in the absence of T cells *in vivo*. These type II T-independent antigens activate B cells by cross-linking the B cell AgR strongly and/or persistently (Brunswick *et al.*, 1988, 1989). *In vitro* antibody responses to these antigens require addition of cytokines (Mond *et al.*, 1980; Pecanha *et al.*, 1991), and thus are not truly independent of additional T cell-derived regulatory signals.

Some antigens that cross-link the B cell AgR less well than the highly repetitive type II T-independent antigens can induce early activation events in B cells in the absence of T cells, but subsequent proliferation and antibody production require T cell-derived cytokines. For example, soluble anti-immunoglobulin (anti-Ig) antibodies or haptens conjugated to sheep red blood cells (SRBCs) are capable of inducing B cells to enter the cell cycle, but do not induce extensive proliferation or differentiation to antibody secretion. The types of cytokines required to promote proliferation and differentiation of the antigen-stimulated B cells depend to some extent on the antigenic stimulus. For example, SRBC-stimulated B cells produce antibody in response to IL-2 plus interferon-γ (IFN-γ) (Leibson *et al.*, 1984), whereas anti-Ig-stimulated B cells require addition of IL-4, IL-5, and either IL-2 or another cytokine (probably IL-6) to produce antibodies (Nakanishi *et al.*, 1983, 1984). Clearly, these types of antigens have a greater requirement for T cell involvement than do the T-independent type II antigens, as they require T cells to make *in vivo* antibody responses. Apparently, the

greater ability of the T-independent type II antigens to induce signaling by the B cell AgR decreases the requirement for cytokines from helper T cells.

In contrast to multivalent antigens, such as virions and antigens that are cell surface proteins, soluble protein antigens are either monovalent or oligovalent. These antigens induce weaker signaling through the B cell AgR (Snow *et al.*, 1986; Myers *et al.*, 1987; Grupp *et al.*, 1987; Wilson *et al.*, 1987), or may not induce any signaling at all in the case of monovalent antigens. The activation of B cells by such antigens requires the presence of helper T cells, and this requirement cannot be replaced by supernatants of activated helper T cells or by mixtures of recombiant cytokines (Noelle and Snow, 1990). Recently, the mechanism of this activation has become clear. On activation, helper T cells express a cell surface protein called CD40 ligand (CD40L), which binds to CD40 on the B cell and delivers a signal to the B cell that can induce early activation even in B cells that have not received any signal through the B cell AgR (DeFranco, 1992c). B cells that are activated in this manner require IL-4 and IL-5 in order to proliferate and differentiate into antibody-secreting cells (Hodgkin *et al.*, 1990; Noelle *et al.*, 1991, 1992). The importance of CD40L/CD40 for B cell activation is illustrated by experiments in which this interaction is blocked experimentally with antibodies against CD40L (Noelle *et al.*, 1992) or with soluble CD40 (Fanslow *et al.*, 1992), or genetically in the human disease X-linked hyper-IgM syndrome, in which T-dependent antibody responses are defective (Aruffo *et al.*, 1993; Allen *et al.*, 1993; Korthauer *et al.*, 1993; DiSanto *et al.*, 1993; Fuleihan *et al.*, 1993).

The antigen specificity of T cell-dependent B cell activation is thought to derive from the role of the B cell AgR in facilitating efficient antigen presentation. Specific antigen uptake via the AgR is 10^3- to 10^4-fold more efficient than fluid-phase uptake of antigen (Lanzavecchia, 1990). Antigen uptake is followed by processing, binding of antigen-derived peptides to class II MHC molecules in endosomes, and then expression of these peptide/class II MHC complexes on the B cell surface (Myers, 1991; Brodsky and Guagliardi, 1991). The consequence of this is that antigen-specific B cells are much more efficient antigen-presenting cells than are B cells with other antigen specificities. Thus, in their capacity as antigen-presenting cells, antigen-specific B cells form conjugates with T cells. The antigen-specific helper T cell recognizes the peptide/MHC class II complex expressed by the B cell and this leads to a tight, long-lasting interaction (Kupfer and Singer, 1989; Vitetta *et al.*, 1989). Once the T cell : B cell conjugate

is formed in this way, the T cell becomes activated and expresses CD40L, which stimulates the B cell through CD40.

AgR-mediated antigen uptake can proceed at very low antigen concentrations and does not require AgR cross-linking (Tony *et al.*, 1985; Lanzavecchia, 1985), indicating that the CD40L-stimulated pathway of B cell early activation can occur in the absence of AgR signaling. Nonetheless, T cell-dependent B cell early activation and AgR induction of early activation are not mutually exclusive and may occur simultaneously for oligomeric protein antigens. Signals initiated by cross-linking the AgR can improve the ability of the B cell to present antigen to T cells (Casten *et al.*, 1985), and this likely reflects the accumulated effects of several mechanisms, including up-regulation of class II MHC molecules (Mond *et al.*, 1981), enhanced affinity of LFA-1 (CD11a/CD18) cell adhesion molecules (Dang and Rock, 1991), and induced expression of the costimulatory molecule B7 (Freedman *et al.*, 1987; Freeman *et al.*, 1989).

In contrast to T-independent responses, additional differentiative events occur during T cell-dependent antibody responses. During T-dependent antibody responses, there is extensive class switching to immunoglobulin isotypes other than IgM, whereas antibody responses to T-independent antigens are dominated by IgM. For example, in the X-linked hyper-IgM syndrome, which is a defect of CD40L, there is a normal or elevated serum IgM level, but very little IgG or IgA. In addition, two important hallmarks of the immune system only occur in T-dependent antibody responses: the induction of memory B cells and affinity maturation due to somatic mutation of Ig variable region gene segments. These differentiative steps are driven by a variety of cytokines, including IL-4, IFN-γ, and TGF-β. Memory induction and hypermutation of Ig genes are thought to occur in the germinal centers of lymph nodes (Linton *et al.*, 1992; Jacob *et al.*, 1991; Berek *et al.*, 1991). Signaling by CD40 plays an important role in promoting the survival and differentiation of germinal center B cells (Liu *et al.*, 1989). Thus, it appears that T cell-derived signals play central roles in class switching, affinity maturation, and memory B cell formation.

The first step in the generation of antibody-secreting cells involves antigen contact with the B cell AgR and its function either in antigen uptake and presentation or as a signal-transducing receptor. This receptor has been extensively studied and we have by far the best understanding of its function of any of the B cell receptors involved in B cell activation.

III. Structure of the B Cell Antigen Receptor

B cells bind antigen via cell surface receptors that are expressed on normal B cells at 10^4 to 10^5 copies per cell. The B cell AgR consists of a noncovalently associated complex between the antigen-binding portion, membrane immunoglobulin (mIg), and a disulfide-linked heterodimer composed of two polypeptides called Ig-α and Ig-β (see Fig. 1). The structure of the B cell AgR has been described in detail in a recent review by Reth (1992), so only the more significant points are summarized here.

The mIgs resemble secreted antibodies in that they are composed of two identical heavy chains and two identical light chains, which are joined by disulfide bonds. The mIgs differ from secreted antibodies in that the heavy chains have a different carboxy terminus, which is generated by alternative mRNA splicing. The exons that are used only

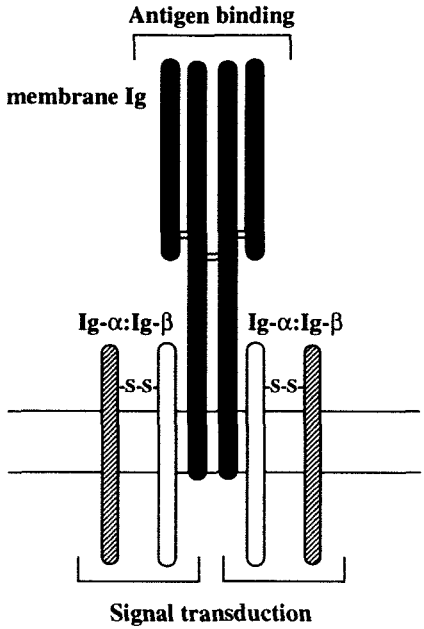

FIG. 1. Structure of the B cell antigen receptor. The B cell AgR consists of an antigen-binding portion (membrane Ig) that is noncovalently associated with disulfide-linked heterodimers consisting of Ig-α and Ig-β. Since the AgR contains two identical Ig heavy chains, it is thought that each AgR contains two Ig-α : Ig-β heterodimers. This has not been confirmed experimentally. Due to the very short cytoplasmic tails of the membrane Ig portion of the AgR, it is likely that signal transduction functions are mediated by the Ig-α : Ig-β moiety of the receptor.

in the membrane forms of the heavy chains encode an extracellular spacer region, a transmembrane domain of about 25 amino acids, and a short cytoplasmic domain (only 3 amino acids for mIgM and mIgD). Each of the heavy chain isotypes is capable of giving rise to both membrane and secreted forms. The amino-terminal portions of the heavy and light chains are highly variable and differ greatly from one B cell to the next. The variable regions of the heavy and light chains together form the antigen-binding pocket of the Ig molecules and each Ig molecule therefore has two identical antigen-binding sites. Immature B cells express exclusively mIgM on their surface and this reflects the fact that the completed heavy chain variable region is initially rearranged just upstream of the μ constant region gene. Mature B cells express simultaneously mIgM and mIgD molecules, which on a single cell have identical antigen-binding sites. The μ and δ constant region genes are closely spaced and can be joined to the same variable region either by alternative splicing of a long transcript or by differential transcriptional termination and splicing. B cell activation gives rise to memory B cells that have undergone class switching and express on their surface mIgG, mIgA, or mIgE. This isotype switch is due to an additional DNA rearrangement that excises the μ and δ constant region genes such that the same completed variable region gene is now upstream of the gene encoding the constant region of one of these isotypes.

Several observations suggested that the mIgs must interact with other proteins in order to form a functional receptor that can be expressed on the cell surface and transmit a signal across the plasma membrane. First, the very short cytoplasmic tails of mIgM and mIgD, only 3 amino acid residues long, effectively preclude these mIgs from interacting directly with cytoplasmic signaling components such as protein kinases. Second, the membrane-spanning region of the μ heavy chain is highly conserved throughout evolution (Reth, 1992), suggesting that there is strong selection against divergence in this region, perhaps because it is required for interaction with other proteins. Of the 25 amino acid residues in the transmembrane domain, 10 contain hydroxyl side chains. This is an unusually high number of polar residues for a membrane-spanning region and suggests that some of these amino acids are involved in interactions with other proteins. Finally, several experiments showed that one or more B cell-specific components are required for transport of mIgM to the cell surface. For example, Reth and colleagues found that in the antibody-secreting plasmacytoma cell line J558, transfected mIgM was not expressed on the cell surface, but was retained in the endoplasmic reticulum (ER)

(Hombach *et al.*, 1988b). Similarly, when mIgM is expressed in fibroblasts or in a pituitary cell line by transfection, it is also retained in the endoplasmic reticulum and not expressed on the cell surface (Williams *et al.*, 1990; Matsuuchi *et al.*, 1992).

The proteins required for transit of mIgM to the cell surface are called Ig-α and Ig-β. These polypeptides were first recognized by Hombach *et al.*, (1988a), who selected a spontaneous variant of the J558 cell line that would express mIgM on the cell surface. By solubilizing the cells in digitonin, a mild detergent that has been used to preserve the noncovalent association of other membrane protein complexes, these workers showed that a disulfide-linked heterodimer specifically co-precipitated with mIgM only in the cells in which mIgM was expressed on the cell surface and not in the parental J558 cells. The association of this disulfide-linked heterodimer with mIgM was subsequently observed in normal B cells and in B lymphoma cell lines from both mouse and human (Campbell and Cambier, 1990; Chen *et al.*, 1990; van Noesel *et al.*, 1991). This noncovalent interaction is maintained when cells are solubilized in mild detergents such as 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), but is disrupted by Triton X-100 and NP-40. The two components of the disulfide-linked heterodimer are termed Ig-α and Ig-β (Reth, 1992). In murine B cells, Ig-α is about 34 kDa and Ig-β is about 38 kDa (Hombach *et al.*, 1990a). Both are extensively glycosylated, which often results in multiple molecular weight forms of each protein. After deglycosylation, murine Ig-α is approximately 24 kDa and Ig-β is 25 kDa (Campbell *et al.*, 1991a). In humans, the deglycosylated forms of Ig-α and of Ig-β are also about 24 kDa; however, Ig-α is glycosylated to a greater extent than in mouse and the mature protein has a molecular mass of about 47 kDa (van Noesel *et al.*, 1991). Since the mIgM molecule contains two identical μ heavy chains, it is has been proposed that each mIgM molecule is associated with two Ig-α : Ig-β heterodimers, although this has not been confirmed experimently.

Direct protein sequencing of the IgM-associated proteins revealed that Ig-α is the product of the *mb-1* gene whereas Ig-β is encoded by the *B29* gene (Hombach *et al.*, 1990a; Campbell *et al.*, 1991a,b). Both of these genes had been cloned previously based on their being expressed only in B lineage cells (Sakaguchi *et al.*, 1988; Hermanson *et al.*, 1988). The *B29* gene is expressed throughout B cell development (Hermanson *et al.*, 1988). In contrast, the *mb-1* gene is turned off in antibody-secreting plasma cells but is expressed in all earlier stages of B cell development (Sakaguchi *et al.*, 1988). This explains the failure of transfected mIgM to be expressed on the cell surface in the J558

plasmacytoma cells (Hombach *et al.*, 1988b). The J558 variant that was able to express mIgM on the cell surface had spontaneously turned on expression of the *mb-1* gene (Hombach *et al.*, 1990b). Transfection of the parental J558 cells with an *mb-1* expression vector also allowed cell surface expression of mIgM that was associated with the disulfide-linked heterodimer (Hombach *et al.*, 1990b). Cotransfection of the J558 cells with the *mb-1* expression vector and expression vectors encoding the membrane forms of the μ, δ, γ2b, α or ε heavy chains showed that all five classes of mIg can associate with the Ig-α : Ig-β heterodimer (Venkitaraman *et al.*, 1991). Although the same Ig-α : Ig-β heterodimer associates with all five classes of Ig heavy chain, the molecular weight of the mature Ig-α chain differs depending on which class of mIg it is associated with. Ig-α associated with mIgD or with mIgA is 1–2 kDa larger than the Ig-α associated with the other classes of mIg (Venkitaraman *et al.*, 1991). This is probably due to differences in glycosylation since the Ig-α chains exhibit identical electrophoretic mobility after deglycosylation (Venkitaraman *et al.*, 1991). The IgD and IgA heavy chains may somehow block the utilization of a glycosylation site on Ig-α. This difference in the molecular weight of the IgM- and IgD-associated Ig-α proteins had previously been seen in murine splenic B cells that express both mIgM and mIgD, and it was initially interpreted as evidence that there are different Ig-α proteins associated with mIgM and mIgD. However, both the IgM- and IgD-associated Ig-α proteins react with a variety of antibodies raised against synthetic peptides based on the *mb-1* DNA sequence (Sakaguchi *et al.*, 1988; Gold *et al.*, 1991), and a CNBr fragment derived from both had the identical sequence (Venkitaraman *et al.*, 1991).

Cambier and colleagues have also detected disulfide-linked heterodimers associated with mIgM that contain an additional polypeptide with a molecular weight that is in between that of Ig-α and Ig-β (Campbell and Cambier, 1990). This polypeptide, which they termed Ig-γ, is expressed predominantly in activated B cells and not in resting cells. Ig-γ is probably a truncated *B29* gene product (Friedrich *et al.*, 1993), although the exact structure of this component has not been determined.

Surface expression of mIgM requires the presence of both Ig-α and Ig-β. Venkitaraman *et al.* (1991) and Matsuuchi *et al.* (1992) have analyzed the requirements for cell surface expression of mIgM by transfecting non-B cells (NIH3T3 cells and the pituitary cell line AtT20, respectively) with genes encoding mIgM, Ig-α, and Ig-β. In the absence of either Ig-α or Ig-β, mIgM is retained intracellularly in the ER and does not progress to the cell surface. In the presence of both

Ig-α and Ig-β, mIgM comes to the cell surface. No other lymphoid-specific components are required for cell surface expression of mIgM in these cells, and it is likely that Ig-α and Ig-β are the only components required for cell surface expression of mIgM. Further work has shown that in the absence of mIgM, Ig-α and Ig-β form a disulfide-linked heterodimer that is retained in the ER (L. Matsuuchi, University of British Columbia, unpublished results). Thus, it appears that all four of the chains of the B cell AgR are required for the receptor to be transported to the cell surface. Incomplete, and therefore nonfunctional, receptors lacking subunits are retained intracellularly and presumably targeted for degradation. Similar observations have been made for the T cell receptor (TCR) and other receptors (Klausner, 1989). Although the basis for this retention is not clearly understood, it most likely involves recognition by ER components of incorrectly folded proteins or exposed hydrophobic patches that would normally interact with other polypeptide chains (deSilva *et al.*, 1990). One component that binds to incompletely assembled mIgM molecules that are retained in the ER is IP-90 (Hochstenbach *et al.*, 1992). One interesting possibility is that IP-90 is a molecular chaperone that facilitates the assembly of mIg with the Ig-α : Ig-β heterodimer.

Whereas all five classes of mIg can associate with the Ig-α:Ig-β heterodimer, mIgD and mIgG_{2b} can be transported to the cell surface in the absence of Ig-α and Ig-β in fibroblasts (Venkitaraman *et al.*, 1991). In contrast, cell surface expression of mIgM, mIgA, and mIgE requires Ig-α and Ig-β. The transmembrane domains of IgD and IgG_{2b} may lack the strong retention signals found in the other Ig heavy chains. It should be emphasized that although mIgD and mIgG_{2b} can come to the surface without Ig-α and Ig-β, Ig-α and Ig-β are expressed at all stages of B cell development in which mIg is also expressed (Sakaguchi *et al.*, 1988; Hermanson *et al.*, 1988). In addition, immunoprecipitation with antibodies against Ig-α removes all of the mIgM and all of the mIgD from lysates of murine splenic B cells (Gold *et al.*, 1991). On this basis, it appears that all of the mIgD that is expressed at the cell surface of splenic B cells is present in a complex with Ig-α : Ig-β. It is possible that in some subset of B cells, mIgD or mIgG_{2b} is expressed in excess of Ig-α and Ig-β and that some fraction of these mIgs are transported to the cell surface without Ig-α and Ig-β. In any case, it is likely that Ig-α and Ig-β mediate signal transduction by the B cell AgR, and thus would be required for most functions of this receptor. This view is supported by experiments showing that chimeric membrane proteins containing a short conserved motif from the cytoplasmic domain of either Ig-α or Ig-β can mediate many of the signaling reactions characteristic of the B cell AgR (Law *et al.*, 1993).

IV. Initial Signaling Reactions Stimulated by the B Cell Antigen Receptor

A. AgR-Induced Protein Tyrosine Phosphorylation

The B cell AgR belongs to the class of receptors that stimulate tyrosine phosphorylation of proteins on activation. Activation of the B cell AgR requires that the receptor be clustered or "cross-linked" by either a multivalent antigen or by antireceptor (anti-Ig) antibodies (Cambier and Ransom, 1987; DeFranco, 1987). A dramatic increase in the tyrosine phosphorylation of a number of proteins is seen within seconds of adding anti-Ig antibodies to B cells. This response has been observed in murine splenic B cells, in human tonsillar B cells, and in a variety of murine and human B cell lines (Gold *et al.*, 1990; Campbell and Sefton, 1990; Lane *et al.*, 1990; Brunswick *et al.*, 1991). When analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with antiphosphotyrosine antibodies, the most prominent anti-Ig-induced tyrosine-phosphorylated proteins have approximate molecular masses of 32–37, 40–45, 50–56, 65, 69–73, 81, 88, 100, 115, 126, 150 kDa (Gold *et al.*, 1990; Campbell and Sefton, 1990; Lane *et al.*, 1990). Tyrosine phosphorylation of these proteins has been confirmed by performing phosphoamino acid analysis on proteins immunoprecipitated with antiphosphotyrosine antibodies (anti-P-tyr) from ^{32}P-labeled cells and demonstrating the presence of phosphotyrosine (Gold *et al.*, 1990; Campbell and Sefton, 1990; DeFranco *et al.*, 1993). Ligation of mIgD on murine splenic B cells appears to induce tyrosine phosphorylation of the same set of proteins as does anti-IgM treatment (Gold *et al.*,1990; Brunswick *et al.*, 1991). Cross-linking of mIgG on murine B cell lines that express this mIg isotype also induces tyrosine phosphorylation, although fewer induced bands are seen on anti-P-tyr immunoblots (Law *et al.*, 1992). This, however, appears to be a property of the cells, and not of the IgG-containing AgR, since the same pattern of tyrosine phosphorylation is elicited by cross-linking of mIgM-containing AgRs in the same cells after transfection of a gene encoding a functional μ chain (Blum *et al.*, 1993).

Tyrosine phosphorylation seen in response to soluble anti-Ig is a transient response. After achieving maximal tyrosine phosphorylation at 3–5 minutes, the level of phosphorylation decreased and returns to near basal levels after 2–4 hours (Gold *et al.*, 1991). Since responses to AgR signaling such as entry into the cell cycle often do not occur until 24–48 hours and require the continued presence of anti-Ig (DeFranco *et al.*, 1985), mIg signaling must continue, although probably at a decreased level.

AgR-induced tyrosine phosphorylation is thought to be due to acti-

vation of tyrosine kinases. A variety of tyrosine kinase inhibitors that act by different mechanisms are capable of abrogating anti-Ig-induced tyrosine phosphorylation (Lane *et al.*, 1991; Carter *et al.*, 1991a; Padeh *et al.*, 1991; Gold *et al.*, 1992b). None of the known chains of the B cell AgR contain kinase activity, and therefore the AgR must interact with distinct kinase molecules. Indeed, the *src*-family kinases $p55^{blk}$, $p53/p56^{lyn}$, $p59^{fyn}$, and $p56^{lck}$ have all been shown to be associated with the B cell AgR (Yamanashi *et al.*, 1991; Burkhardt *et al.*, 1991; Campbell and Sefton, 1992; Lin and Justement, 1992; Leprince *et al.*, 1992; Li *et al.*, 1992). Burkhardt *et al.* (1991) showed that AgR cross-linking results in rapid activation of $p55^{blk}$, $p53/56^{lyn}$, and $p59^{fyn}$ as judged by an increase in the ability of the immunoprecipitated kinases to carry out autophosphorylation and to phosphorylate an exogenous substrate, enolase, in an *in vitro* kinase assay. In murine splenic B cells, mIgD cross-linking causes a five- to eight-fold increase in the activity of $p55^{blk}$ that is maximal at 0.5–1 minute and remains above basal levels for at least 15 minutes (Burkhardt *et al.*, 1991). Sustained activation of $p59^{fyn}$ is also observed, whereas only a small, transient increase in the activity of $p53/p56^{lyn}$ is seen (Burkhardt *et al.*, 1991). These findings suggest that $p55^{blk}$, the only one of these kinases expressed exclusively in B cells (Dymecki *et al.*, 1990), may make the largest contribution to AgR-stimulated tyrosine phosphorylation in splenic B cells. Yamanishi *et al.*, however, did observe a substantial AgR-induced activation of $p53/p56^{lyn}$ in the murine B lymphoma line WEHI-231 and in the human B cell line Daudi (Yamanashi *et al.*, 1992). $p56^{lck}$ is also activated by AgR cross-linking, at least in the murine B cell line WEHI-231 (Li *et al.*, 1992; R. Chiu, R. Ingham, and M. R. Gold, unpublished observations). Hutchcroft *et al.* have shown that the prominent 72-kDa tyrosine-phosphorylated protein seen in anti-P-tyr blots of activated B cells is a tyrosine kinase (PTK72) that associates with the B cell AgR and is activated on AgR cross-linking (Hutchcroft *et al.*, 1991, 1992). PTK72 is probably the product of the *syk* gene (Taniguchi *et al.*, 1991), which encodes a putative 72-kDa tyrosine kinase. *syk* is not related to the *src*-family tyrosine kinases but has homology to the T cell tyrosine kinase ZAP70, which associates with the ζ chain of the T cell receptor (Chan *et al.*, 1992). Thus, the B cell AgR associates with and activates at least five different tyrosine kinases. The functions of the individual kinases, the mechanism by which they are activated, and the nature of their interactions with the B cell AgR are poorly understood at this point (see later).

Stimulation of protein tyrosine phosphorylation by the B cell AgR is significant in light of the strong correlation between tyrosine phosphorylation, receptor signaling, and control of cell growth and differ-

entiation in other cell types. Many receptors stimulate tyrosine phosphorylation either by virture of having an intrinsic tyrosine kinase activity [e.g., the platelet-derived growth factor receptor (PDGF-R) and the epidermal growth factor receptor (EGF-R)] or by their ability to activate intracellular tyrosine kinases. This latter class includes the T cell receptor, the hematopoietin receptors such as those for IL-3, IL-4, IL-5, IL-7, and GM-CSF, as well as FcεRI and FcγRIII (CD16) (Keegan and Paul, 1992; Cambier, 1992; Samelson and Klausner, 1992; Miyajima *et al.*, 1992; Kolanus *et al.*, 1992). A large number of the known oncogene products are also tyrosine kinases. Furthermore, as discussed later, many of the known substrates of tyrosine kinases are important signal transduction molecules (Cantley *et al.*, 1991). Finally, many important signaling components contain structural motifs called *src* homology 2, or SH2, domains (Koch *et al.*, 1991; Songyang *et al.*, 1993), which function as specific receptors for phosphotyrosine-containing regions of proteins. In this manner, tyrosine phosphorylation facilitates the interaction of proteins involved in signal transduction, presumably making the process more efficient.

B. Targets of AgR-Induced Tryosine Phosphorylation

If tyrosine phosphorylation is indeed an important mediator of the effects of AgR stimulation on B cells, then to understand this process, it is essential to identify the substrates of the AgR-activated tyrosine kinases and to understand their roles in the signal transduction process. Two strategies have been employed to identify tyrosine kinase substrates in B cells. One involves purification of these proteins by anti-P-tyr affinity chromatography. Proteins eluted with phosphotyrosine analogs from these columns are separated by SDS-PAGE, individual protein bands are excised and digested with proteases, and the resulting peptides are subjected to protein microsequencing. Amino acid sequences of these peptides could reveal the identity of the protein, provided the gene encoding it has been cloned and its sequence is in the existing data bases. If the gene has not been cloned, degenerate oligonucleotides based on the peptide sequence can be used to amplify a fragment of cDNA by polymerase chain reaction and this can then be used to screen cDNA libraries. Alternatively, the purified tyrosine kinase substrate can be used to raise antibodies, which can then be used to screen expression libraries and clone the gene encoding the tyrosine kinase substrate (Schulte *et al.*, 1992).

A second approach is to immunoprecipitate known tyrosine kinase substrates with specific antibodies and then determine whether AgR cross-linking increases their tyrosine phosphorylation. This approach

has yielded considerable information to date and has been facilitated by extensive work characterizing substrates of receptors with intrinsic tyrosine kinase activity such as the PDGF-R and EGF-R. Ligand binding by these receptors induces the receptors to dimerize, which activates the tyrosine kinase contained in the cytoplasmic tails of these receptors. The dimerized receptors transphosphorylate each other on tyrosine residues at multiple sites. This creates distinct specific binding sites for several important signaling molecules that bind to the receptor via SH2 domains. After binding to the receptors, these signaling components are then tyrosine phosphorylated, presumably by the kinase activity of the receptor. Tyrosine phosphorylation of these proteins may signficantly alter their activity or function. Among the proteins that bind to the PDGF-R and EGF-R are proteins involved in three different signal transduction pathways—phospholipase Cγ, the *ras* GTPase-activating protein (*ras*GAP), and phosphatidylinositol 3-kinase (PI 3-kinase). Although these pathways may intersect, they can be activated independently. For example, the PDGF-R and EGF-R activate all three of these pathways whereas the CSF-1-R activates only the *ras*GAP and PI 3-kinase pathways (Cantley *et la.*, 1991). Activating different combinations of these pathways could produce distinct results or allow for synergistic effects. In T cells, the T cell antigen receptor activates phospholipase C (Imboden and Stobo, 1985) and the $p21^{ras}$ pathway (Downward *et al.*, 1990a) whereas the IL-2 receptor activates PI 3-kinase (Remillard *et al.*, 1991; Abraham *et al.*, 1992). The B cell AgR activates all three of these signaling pathways, making it similar to the PDGF-R.

C. Targets of mIg-Induced Tyrosine Phosphorylation

1. Phospholipase C

Although AgR-induced tyrosine phosphorylation was first reported in 1990, it had been known since 1986 that AgR cross-linking activates the phosphoinositide signaling pathway (Cambier *et al.*, 1987; DeFranco, 1992b), in which the initial event is activation of phospholipase C (PLC). Phospholipase C catalyzes the hydrolysis of inositol-containing phospholipids, primarily phosphatidylinositol 4,5-bisphosphate (PIP_2). This results in the generation of two intracellular second messengers: (1) inositol 1,4,5-trisphosphate (IP_3), which causes increases in cytosolic free calcium concentrations by stimulating the release of calcium from intracellular storage vesicles, and (2) diacylglycerol, which activates the α, β and γ subtypes of the serine/threonine kinase protein kinase C (PKC). IP_3 is acted on by kinases and

phosphatases to produce a large number of other inositol phosphate isomers. It is unclear whether these other inositol phosphate compounds have signaling functions, although there is some evidence that inositol 1,3,4,5-tetrakisphosphate contributes to increases in cytosolic free calcium concentrations (Irvine and Moor, 1986; Morris *et al.*, 1987). Increases in cytosolic free calcium concentrations and increases in inositol phosphate production have been observed in B cells stimulated with either anti-Ig antibodies or with multivalent antigens (Grupp *et al.*, 1987; Wilson *et al.*, 1987). PKC activation results in translocation of the enzyme from the cytosol to the plasma membrane. AgR signaling has been shown to cause a transient increase in the fraction of PKC associated with the plasma membrane (Nel *et al.*, 1986; Chen *et al.*, 1986), and several known PKC substrates, including the MARCKS protein (Aderem, 1992), are phosphorylated in response to AgR cross-linking (Hornbeck and Paul, 1986; Hornbeck *et al.*, 1989).

When it was discovered that the B cell AgR also stimulated protein tyrosine phosphorylation, the question arose as to how these two signaling pathways were related. A number of experiments demonstrated that tyrosine phosphorylation is not dependent on activation of the phosphoinositide pathway. Tyrosine phosphorylation could not be stimulated by activating PKC with phorbol esters, by elevating intracellular calcium concentrations with calcium ionophores, or by the combination of phorbol esters and calcium ionophores (Gold *et al.*, 1990). The main exception is a 42-kDa protein (mitogen-activated protein kinase; see below) whose tyrosine phosphorylation can be induced by PKC activation (Casillas *et al.*, 1991; Gold *et al.*, 1992a). To rule out the possibility that other PIP_2-derived second messengers (e.g., inositol phosphate isomers besides IP_3) activate tyrosine kinases, another receptor that activates PLC, the M1 muscarinic acetylcholine receptor, was introduced into B cell lines by transfection. Stimulation of this transfected muscarinic receptor with the agonist carbachol leads to inositol phosphate production, but stimulates tyrosine phosphorylation only of the same 42-kDa protein whose tyrosine phosphorylation can be stimulated by phorbol esters (Gold *et al.*, 1992a; Mittelstadt and DeFranco, 1993). Thus AgR-induced tyrosine phosphorylation is not due to activation of PLC. Furthermore, depletion of PKC by prolonged treatment of cells with phorbol esters does not significantly reduce anti-Ig-stimulated tyrosine phosphorylation nor does inhibition of PLC activation by acute treatment of cells with high concentrations of phorbol esters (Brunswick *et al.*, 1991). Tyrosine kinase inhibitors, however, block not only AgR-induced tyrosine phosphorylation, but also AgR-stimulated phosphoinositide breakdown and calcium eleva-

tion (Lane *et al.*, 1991; Carter *et al.*, 1991a; Padeh *et al.*, 1991). Thus, phospholipase C activation is dependent on tyrosine phosphorylation and it is likely that tyrosine kinase activation is the initial signaling event stimulated by the B cell AgR.

Several groups have now shown that AgR cross-linking stimulates tyrosine phosphorylation of two isozymes of PLC in B cells, PLC-γ1 and PLC-γ2 (Carter *et al.*, 1991a; Hempel *et al.*, 1992; Coggeshall *et al.*, 1992; Kanner *et al.*, 1992; Roifman and Wang, 1992). Tyrosine phosphorylation activates PLC-γ1 by overcoming the inhibitory effects of profilin (Goldschmidt-Clermont *et al.*, 1991). Profilin is an abundant cytoskeletal protein that binds to PIP_2. In the presence of profilin, PLC-γ1 is incapable of hydrolyzing PIP_2 unless it has been tyrosine phosphorylated. Given the extensive homology of PLC-γ2 to PLC-γ1, it seems likely that these two PLC isoforms are regulated in a similar manner.

Regulation of PLC by the B cell AgR may be more complex than this simple model. There is considerable evidence that AgR-induced inositol phosphate production involves a GTP-binding protein (Gold *et al.*, 1987; Harnett and Klaus, 1988; Page *et al.*, 1991) analogous to the G proteins that mediate signaling by receptors with seven transmembrane domains, such as the β-adrenergic receptor (Bourne *et al.*, 1991). The involvement of such proteins in AgR signaling has been inferred from studies using permeabilized cells in which it was shown that stable GTP analogs (which lock receptor-activated G proteins in their active state) potentiate anti-Ig-induced inositol phosphate production whereas GDP analogs (which prevent the activation of G proteins by competing for the GTP-binding site) inhibit inositol phosphate production (Gold *et al.*, 1987; Harnett and Klaus, 1988). The identity of this GTP binding is not known; it is not clear whether it is a heterotrimeric G protein or a small GTP-binding protein such as $p21^{ras}$.

There are two models that could explain the dual requirement of tyrosine phosphorylation and G protein activation for PLC activation by the AgR. One possibility is that activation of PLC-γ1 and PLC-γ2 in B cells requires both tyrosine phosphorylation of the enzyme and its interaction with an activated G protein. Such combinatorial regulation has been observed for activation of PLC-γ1 by the EGF-R in hepatocytes (Yang *et al.*,1991). Alternatively, the B cell AgR could activate PLC-γ1 and PLC-γ2 via tyrosine phosphorylation while activating another PLC isozyme in a G protein-dependent manner. For example, PLC-β can be activated *in vitro* by the G protein Gq (Taylor *et al.*, 1991). Murine B cells do not express PLC-β1 (Hempel and DeFranco, 1991) but apparently express another G protein-regulated PLC iso-

zyme, since the M1 muscarinic acetylcholine receptor, which has been shown to signal via a heterotrimeric G protein (Ashkenazi *et al.*, 1989), can trigger PIP_2 breakdown in B cell lines (see previous discussion). Since tyrosine kinase inhibitors block AgR-induced PLC activation, one must postulate that either the G protein-dependent pathway is a minor pathway or that activation of the G protein (or its ability to regulate PLC) is dependent on tyrosine phosphorylation in some way.

The PIP_2-derived second messengers appear to play an important role in mediating the effects of AgR cross-linking on B cells. Increases in intracellular calcium concentrations presumably lead to activation of calcium/calmodulin-dependent protein kinases such as calmodulin-dependent protein kinase II. PKC phosphorylates a number of proteins and also initiates protein kinase cascades that lead to the phosphorylation and activation of mitogen-activated protein kinase (see later) as well as proteins involved in regulating gene expression, such as c-Jun (Boyle *et al.*, 1991; Pulverer *et al.*, 1991). c-Jun is part of the AP-1 transcription factor that binds PKC-responsive promoter elements and activates transcription from them (Ransone and Verma, 1990). Several lines of evidence suggest that these signaling events are essential for responses to AgR engagement. The combination of phorbol esters and calcium ionophores can mimic many of the effects of AgR cross-linking, including the induction of immediate early genes such as c-*myc*, c-*fos*, and *egr-1* (Klemsz *et al.*, 1989; Seyfert *et al.*, 1990) (see below) and the entry of resting B cells into the cell cycle (Monroe and Kass, 1985; Klaus *et al.*, 1986; Rothstein *et al.*, 1986; Paul *et al.*, 1986). Inhibition of PKC with pharmacologic inhibitors, as well as down-regulation of PKC by long-term exposure to phorbol esters, blocks many of the responses to anti-Ig antibodies (Monroe and Kass, 1985; Mond *et al.*, 1987). Moreover, culturing resting B cells in low-calcium medium prevents a sustained increase in intracellular calcium and also inhibits anti-Ig-induced proliferation (Dennis *et al.*, 1987). LPS-induced proliferation is unaffected by low extracellular calcium, arguing that this is a specific inhibition of anti-Ig-induced proliferation (Dennis *et al.*, 1987). Additional support for a role for calcium elevation in the response to AgR cross-linking has come from studies using the WEHI-231 B cell line. This cell line undergoes growth arrest in the G_1 phase of the cell cycle followed by programmed cell death in response to anti-IgM antibodies (Page and DeFranco, 1990; Benhamou *et al.*, 1990; Hasbold and Klaus, 1990). Several mutants selected for resistance to anti-Ig-induced growth arrest exhibit reduced anti-Ig-stimulated inositol phosphate production compared to wild-type cells (Page *et al.*, 1991). Normal anti-Ig-induced growth arrest could be

restored by addition of the calcium ionophore ionomycin, but not by addition of phorbol esters. These observations argue that calcium is the limiting second messenger in these cells and is therefore an essential mediator of the response to AgR cross-linking.

2. *p21^{ras}, rasGAP, and rasGAP-Associated Proteins*

The p21^{ras} protooncogene product is thought to play an important role in mediating the action of tyrosine kinases. For example, microinjection of anti-Ras antibodies into fibroblasts blocks growth factor-stimulated proliferation and transformation by tyrosine kinase oncogenes (Mulcahy *et al.*, 1985; Smith *et al.*, 1986). In addition, there is genetic evidence in both *Drosophila* (Rubin, 1991) and *Caenorhabditis elegans* (Sternberg and Horvitz, 1991) that functional *ras* is required for tyrosine kinase receptors to exert their effects. p21^{ras} is a membrane-associated GTP-binding protein. The GTP-bound state of p21^{ras} is presumed to be the active form since transforming versions of p21^{ras} have greatly decreased GTPase activity (McGrath *et al.*, 1984; Gibbs *et al.*, 1984), and signaling by a variety of growth factors increases the fraction of cellular p21^{ras} with GTP bound (Satoh *et al.*, 1990a,b; Duronio *et al.*, 1992). When the B cell AgR is cross-linked with anti-Ig antibodies, p21^{ras} can be shown to cocap with the AgR Graziadei *et al.*, 1990). It is not clear if this reflects activation of p21^{ras}, and it is not known whether p21^{ras} interacts with the AgR directly or via intervening proteins. Delovitch and colleagues (Lazarus *et al.*, 1993) have shown that AgR signaling increases the fraction of p21^{ras} with bound GTP in an antigen-specific B cell line. Thus, p21^{ras}, a potent regulator of cell growth, is activated by the B cell AgR.

The mechanism by which tyrosine kinases regulate p21^{ras} is not completely understood. p21^{ras} activity can be controlled either by guanine nucleotide exchange proteins that promote the binding of GTP to p21^{ras} or by GTPase-activating proteins (GAPs) that favor the inactive form of p21^{ras} by promoting the hydrolysis of bound GTP to GDP. Guanine nucleotide exchange factors for p21^{ras} have been identified (Downward *et al.*, 1990b; Bonfini *et al.*, 1992; Martegani *et al.*, 1992) but it has not been shown experimentally that their activity is regulated by tyrosine kinases. Recent work has suggested a model for how tyrosine kinase receptors may activate guanine nucleotide exchange factors for p21^{ras}. Tyrosine kinase activation in fibroblasts induces tyrosine phosphorylation of a protein called SHC (Pelicci *et al.*, 1992). A 23-kDa nonphosphorylated protein called GRB-2 then binds to the SHC protein via its SH2 domain (Rozakis-Adcock *et al.*, 1992). GRB-2 is the mammalian homologue of the product of the *C. elegans*

sem-5 gene (Lowenstein *et al.*, 1992) and the *Drosophila melanogaster drk* gene (Simon *et al.*, 1993; Olivier *et al.*, 1993). *sem-5* and *drk* are required for activation of $p21^{ras}$ by tyrosine kinase receptors involved in vulval development in *C. elegans* (S. G. Clark *et al.*, 1992) and in photoreceptor development in *Drosophila* (Simon *et al.*, 1993; Olivier *et al.*, 1993). The *drk* gene product can bind to both the Sevenless transmembrane tyrosine kinase and to the Sos guanine nucleotide exchange protein (Simon *et al.*, 1993; Olivier *et al.*, 1993). The former interaction seems to be via the SH2 of Drk and to be regulated by receptor autophosphorylation, whereas the latter interaction probably involves the SH3 domains of Drk. These results suggest the Sem-5/Drk/GRB-2 binds to a guanine nucleotide exchange factor, activates it, and in this way regulates the activation state of $p21^{ras}$. This pathway may be relevant to the mechanism by which the B cell AgR regulates $p21^{ras}$, as AgR cross-linking induces tyrosine phosphorylation of SHC in several B cell lines (I. van Oostveen, T. M. Saxton, and M. Gold, unpublished results).

Two GTPase-activating proteins have been identified for $p21^{ras}$—*ras*GAP (Trahey and McCormick, 1987; Gibbs *et al.*, 1988) and neurofibromin, the product of the *NF-1* gene (Downward, 1991). The ability of *ras*GAP to stimulate the GTPase activity of $p21^{ras}$ has been studied extensively (Trahey and McCormick, 1987; Gibbs *et al.*, 1988). *ras*GAP is a target of tyrosine phosphorylation by a number of growth factor receptors (Cantley *et al.*, 1991) and is also tyrosine phosphorylated in response to AgR cross-linking in murine and human B cell lines (Gold *et al.*, 1993a) and in murine splenic B cells (M. Crowley and A. DeFranco, unpublished observations). There is no evidence, however, that tyrosine phosphorylation of *ras*GAP alters its ability to stimulate $p21^{ras}$ GTPase activity. AgR cross-linking in B cells also increases the association of *ras*GAP with two tyrosine-phosphorylated proteins, p62 and p190 (Gold *et al.*, 1993a). Proteins of similar molecular mass are also associated with *ras*GAP in fibroblasts after tyrosine kinase activation (Ellis *et al.*, 1990). A large increase in the amount of tyrosine-phosphorylated p62 associated with *ras*GAP is induced by AgR signaling. In contrast, *ras*GAP is associated with tyrosine-phosphorylated p190 to some extent in unstimulated B cells and the increase induced by AgR signaling is much more variable from cell line to cell line. *ras*GAP binds to tyrosine-phosphorylated p62 and p190 via its SH2 domains (Wong *et al.*, 1992), so a likely scenario is that AgR signaling stimulates tyrosine phosphorylation of p62 and p190 and that this leads to their association with *ras*GAP. It is not clear if the interaction of *ras*GAP with these proteins regulates the GTPase-stimulating activity

of *ras*GAP *in vivo* or if the association of p62 and p190 with *ras*GAP now allows these proteins to somehow influence $p21^{ras}$ activity. *ras*GAP appears to form separate complexes with p62 and p190 (Moran *et al.*, 1991), suggesting that the interaction of *ras*GAP with these two proteins could have distinct effects. A large fraction of p62 is membrane bound (Moran *et al.*, 1991) and the interaction of *ras*GAP with p62 may direct it to the plasma membrane, where $p21^{ras}$ resides. In contrast, it has been reported that the p190–*ras*GAP complex has decreased GTPase-stimulating activity for $p21^{ras}$ *in vitro* (Moran *et al.*, 1991). Clearly, much remains to be elucidated about how $p21^{ras}$ is regulated *in vivo*, and the significance of AgR-induced tyrosine phosphorylation of *ras*GAP, p62, and p190.

Even though activated $p21^{ras}$ has very potent effects on cell growth and differentiation, little is known about the downstream signaling events that are regulated by *ras*. A number of experiments have suggested that *ras*GAP may be a downstream effector of $p21^{ras}$, as well as a regulator of $p21^{ras}$. In several systems, *ras*GAP has been shown to stimulate signaling reactions in a manner that requires the presence of $p21^{ras}$, but not the ability of *ras*GAP to stimulate $p21^{ras}$ GTPase activity (Yatani *et al.*, 1990; Dominguez *et al.*, 1991). Furthermore, in one *in vitro* system, a truncated version of *ras*GAP that lacks the domain required for stimulation of $p21^{ras}$ GTPase activity is active even in the absence of $p21^{ras}$ (Yatani *et al.*, 1990). This strongly supports the contention that *ras*GAP is a downstream effector of $p21^{ras}$ and that $p21^{ras}$ induces conformational changes in *ras*GAP that allow it to act on some downstream element. $p21^{ras}$ has also been implicated in the activation of MAP kinase (Leevers and Marshall, 1992), a serine/threonine kinase described in more detail below.

The genes encoding p62 and p190 have been cloned by Settleman *et al.* (1992b) and Wong *et al.* (1992) and their sequences suggest that they could be downstream elements that are regulated by *ras*GAP. p62 binds RNA *in vitro* and has sequences characteristic of hnRNP proteins that are involved in mRNA splicing (Wong *et al.*, 1992). Thus, tyrosine phosphorylation of p62 and its interaction with *ras*GAP could possibly regulate mRNA splicing, transport of RNA out of the nucleus, translation, or RNA stability. p190 has homology to a transcriptional repressor of the glucocorticoid receptor gene and therefore could be involved in gene regulation (Settleman *et al.*, 1992b). Consistent with such a role, p190 has been detected in the nuclei of rat fibroblasts (Settleman *et al.*, 1992b). p190 has also been shown to stimulate the GTPase activity, and is therefore a "GAP" for two other members of the *ras* family, small G proteins called Rac and Rho (Settleman *et al.*,

1992a). Rac and Rho regulate the formation of actin-based cytoskeletal elements such as stress fibers in fibroblasts (Ridley and Hall, 1992; Ridley *et al.*, 1992).

3. *Phosphatidylinositol 3-kinase*

Another common sequel to tyrosine kinase activation is stimulation of PI 3-kinase, an enzyme that phosphorylates inositol phospholipids on the 3-position of the inositol ring. PI 3-kinase consists of an 85-kDa regulatory subunit that contains two SH2 domains and a 110-kDa catalytic subunit, both of which have now been cloned (Escobedo *et al.*, 1991; Otsu *et al.*, 1991; Skolnik *et al.*, 1991; Hiles *et al.*, 1992). PI 3-kinase phosphorylates phosphatidylinositol, phosphatidylinositol 4-phosphate (PI 4-P), and phosphatidylinositol 4,5-bisphosphate (PI 4,5-P_2) *in vitro* to yield PI 3-P, PI 3,4-P_2, and PI 3,4,5-P_3, respectively. *In vivo*, PI 4,5-P_2 may be the preferred substrate for PI-3 kinase, at least in neutrophils (Stephens *et al.*, 1991). The products of PI 3-kinase are distinct from those of the classical phosphoinositide pathway in which PLC preferentially cleaves PI 4,5-P_2. They are also present at much lower levels than PI 4-P and PI 4,5-P_2. *In vitro* experiments have shown that the PI 3-kinase products are not substrates for PLC (Serunian *et al.*, 1989) and there is currently no evidence that they are cleaved by other phospholipases to yield second messengers. It has been postulated that the lipid products of PI 3-kinase may act as second messengers. Indeed, recently PI 3,4,5-P_3, and (less potently), PI 3,4-P_2 have been shown to activate the ζ form of PKC *in vitro* (Nakanishi *et al.*, 1993). PKC ζ differs from the classical PKC isoforms, α, β, and γ, in that it is not activated by diacylglycerol or phorbol esters (Nakanishi and Exton, 1992) and that it is not down-regulated by chronic treatment with phorbol esters (Liyange *et al.*, 1992). Thus, PI 3-kinase and PKC ζ potentially comprise a new signal transduction pathway.

Although the functions of the PI 3-kinase products are poorly understood, there is considerable evidence that PI 3-kinase is an important downstream mediator of tyrosine kinase action. PI 3-kinase is activated by many tyrosine kinase receptors (e.g., receptors for PDGF, EGF, CSF-1, insulin, and nerve growth factor), by other receptors that activate Src-like tyrosine kinases (e.g., IL-2 receptor), as well as by transforming versions of nonreceptor tyrosine kinases (e.g., v-src, v-abl) (reviewed in Auger and Cantley, 1991). In addition, PI 3-kinase binds directly to many activated tyrosine kinases via its SH2 domains. For example, ligand-induced phosphorylation of the PDGF-R creates specific binding sites that are recognized by the SH2 domains of the p85 regulatory subunit of PI 3-kinase (Fantl *et al.*, 1992; Kazlauskas *et al.*,

1992). This binding is thought to lead to tyrosine phosphorylation and activation of PI 3-kinase. The importance of PI 3-kinase as a mediator of tyrosine kinase action is underscored by studies of mutant tyrosine kinases. Mutant versions of $pp60^{v\text{-}src}$, the polyoma virus middle T protein, and the v-*abl* oncogene product that have lost the ability to bind PI-3 kinase do not transform cells (Auger and Cantley, 1991). Similarly, mutated versions of the PDGF-R that cannot bind PI 3-kinase are incapable of stimulating cell division (Fantl *et al.*, 1992). In contrast, mutations that destroy the binding sites on the PDGF-R for *ras*GAP do not abrogate the ability of the PDGF-R to cause cell division (Fantl *et al.*, 1992). Thus, PI 3-kinase plays a unique and critical role in signal transduction by the PDGF-R and is likely to be important for signaling by other receptors as well.

In B cells, AgR cross-linking causes a large increase in the amount of PI 3-kinase activity that can be immunoprecipitated with anti-P-tyr antibodies (Gold *et al.*, 1992b; Yamanashi *et al.*, 1992). The simplest interpretation of this result is that AgR signaling stimulates tyrosine phosphorylation of PI 3-kinase. This has now been confirmed by immunoprecipitation with antibodies directed against the 85-kDa subunit (p85) of PI 3-kinase followed by immunoblotting with anti-P-tyr. AgR signaling stimulates tyrosine phosphorylation of both the p85 regulatory subunit of the enzyme and the 110-kDa catalytic subunit (Gold *et al.*, 1993b).

Anti-P-tyr immunoblots of anti-PI 3-kinase immunoprecipitates show that AgR signaling induces PI 3-kinase to associate with a number of other tyrosine-phosphorylated proteins in B cells. Since PI 3-kinase associates with many activated tyrosine kinases via its SH2 domains, it is attractive to think that it associates with one of the tyrosine kinases that are activated by the B cell AgR. Yamanashi *et al.* (1992) found that AgR cross-linking induced PI 3-kinase to associate with $p53/p56^{lyn}$ in the WEHI-231 and Daudi cell lines. Although there is a severalfold increase in the amount of PI 3-kinase associated with $p53/p56^{lyn}$, only a very small fraction of the PI-3 kinase (approximately 3% of the amount immunoprecipitated by anti-P-tyr) is associated with $p53/p56^{lyn}$ in anti-Ig-stimulated WEHI-231 cells (I. van Oostveen and M. R. Gold, unpublished observations). Following anti-Ig stimulation, PI 3-kinase can also be seen to associate with the cytoplasmic tail of CD19 (Tuveson *et al.*, 1993). CD19 is a B cell-specific transmembrane protein that is associated with complement receptor 2 (CR2) and two other polypeptides, TAPA-1 and Leu-13 (Bradbury *et al.*, 1992). In addition, some CD19 can be found associated with the B cell AgR (Pesando *et al.*, 1989). Moreover, anti-Ig stimulation induces tyrosine

phosphorylation of CD19 (Tuveson *et al.*, 1993). As the cytoplasmic tail of CD19 has several YxxM motifs that have been identified as the binding site for the SH2 domain of PI 3-kinase p85 (Songyang *et al.*, 1993), an attractive possibility is that anti-Ig induces tyrosine phosphorylation of these tyrosines. This would lead to binding of PI 3-kinase and then tyrosine phosphorylation of PI 3-kinase by a tyrosine kinase associated either with the AgR or with CD19. This possible mechanism is similar to that proposed for activation of PI 3-kinase by growth factor receptors, the difference in those cases being that YxxM is an autophosphorylation site on the receptor (Cantley *et al.*, 1991; Fantl *et al.*, 1992).

AgR-induced activation of PI 3-kinase activity in the intact B cell has been demonstrated by high-performance liquid chromatography (HPLC) analysis of inositol-containing phospholipids in ^{32}P-labeled B cells. Stimulation of WEHI-231 cells with anti-Ig antibodies causes a rapid increase in the amounts of labeled PI 3,4,5-P_3 and PI 3,4-P_2 (Gold *et al.*, 1993b). Maximal increases are seen at 0.5–2 minutes and PI 3,4-P_2 increases to a much greater extent than does PI 3,4,5-P_3. A slower, sustained increase in the amount of labeled PI 3-P is also seen. The total radioactivity in all lipids with phosphates on the 3-position of the inositol ring is increased, demonstrating that PI 3-kinase is activated by AgR cross-linking in these cells. The mechanism by which PI 3-kinase is activated is still poorly understood, but tyrosine phosphorylation and/or binding to other proteins, such as p53/p56^{lyn} and CD19, may be important.

4. *p95^{vav} and Vap-1*

Another target of AgR-induced tyrosine phosphorylation is the protooncogene product p95^{vav}, which is expressed only in hematopoietic cells (Bustelo and Barbacid, 1992). Anti-Ig stimulation of B cells leads to increased tyrosine phosphorylation of p95^{vav} and appearance of a 70-kDa tyrosine-phosphorylated protein (called Vap-1, or Vav-associated protein 1) that is coprecipitated with p95^{vav} only after AgR stimulation (Bustelo and Barbacid, 1992). Vap-1 is transiently tyrosine phosphorylated after anti-Ig-stimulation, and the binding of p95^{vav} to Vap-1 is mediated by the SH2 domain of p95^{vav} binding to phosphotyrosine on Vap-1 (Bustelo and Barbacid, 1992). N-terminal deletions in p95^{vav} render it oncogenic, suggesting that the wild-type p95^{vav} protein may play a regulated role in controlling cell growth. In addition to containing an SH2 domain, p95^{vav} also has a zinc finger motif similar to the diacylglycerol-binding domain of PKC. Moreover, it has significant amino acid homology to the product of the CDC24 gene

from *Saccharomyces cerevisiae*, the product of the *dbl* protooncogene, and the *bcr* gene. As CDC24 encodes a guanine nucleotide exchange factor for the *S. cerevisiae* homologue of Rho, it is possible that $p95^{vav}$ regulates the activity of a small GTP-binding protein such as $p21^{ras}$ or Rho. Recent evidence suggests that $p95^{vav}$ has guanine nucleotide exchange factor activity for $p21^{ras}$ (Gulbins *et al.*, 1993). Moreover, tyrosine phosphorylation is apparently necessary to activate this function. Thus, AgR-induced tyrosine phosphorylation of $p95^{vav}$ may be an important event for regulating $p21^{ras}$.

5. *Mitogen-Activated Protein Kinase*

The mitogen-activated protein (MAP) kinases (also called ERKs, for extracellular signal-regulated kinases) are a family of related serine/threonine kinases, of which the best studied in mammalian cells are the 44-kDa ERK1 ($p44^{mapk}$) and the 42-kDa ERK2 ($p42^{mapk}$). These MAP kinases are activated and tyrosine phosphorylated in response to signaling by many receptors that activate either tyrosine kinases or PKC (Cobb *et al.*, 1991; Pelech and Sanghera, 1992). In B cells, both AgR cross-linking and PKC activation result in tyrosine phosphorylation and activation primarily of ERK2 ($p42^{mapk}$) (Casillas *et al.*, 1991; Gold *et al.*, 1992a). ERK2 is the most prominent tyrosine-phosphorylated protein seen in anti-P-tyr blots of WEHI-231 cells stimulated with anti-Ig antibodies (Gold *et al.*, 1990); in these cells anti-Ig stimulation causes a transient 15- to 20-fold increase in MAP kinase activity that peaks after 3–5 minutes (Gold *et al.*, 1992a).

The mechanism by which MAP kinases are activated is now the subject of intense scrutiny (see Pelech and Sanghera, 1992). Both threonine and tyrosine phosphorylation of ERK1 and ERK2 are required for maximal activation. Both of these modifications are carried out by a dual-specificity (serine/threonine/tyrosine) kinase termed MAP kinase kinase, or MEK (for MAP kinase or ERK kinase). MAP kinase kinase is regulated by phosphorylation also, but, in contrast to MAP kinase, it appears to be activated by serine/threonine phosphorylation, not by tyrosine phosphorylation. This suggests that PKC could activate MAP kinase kinase directly whereas tyrosine kinases must activate an intermediary serine kinase. It has been shown that the serine kinase Raf-1 can activate MAP kinase kinase (Dent *et al.*, 1992; Kyriakis *et al.*, 1992). Raf-1 is activated by both tyrosine kinases (Morrison *et al.*, 1989) and by PKC (Siegel *et al.*, 1990) in different systems. This raises the possibility that it is Raf-1 that integrates signals emanating from tyrosine kinases and PKC, resulting in activation of MAP kinase.

The mechanism of activation of Raf-1 by tyrosine kinases is not well understood. There is considerable evidence that $p21^{ras}$ is involved in the activation of Raf-1 (reviewed in Roberts, 1992). In addition, a second pathway for activating MAP kinase kinase has recently been described that utilizes a G protein and is independent of Raf-1 (Lange-Carter *et al.*, 1993). In any case, it is clear that MAP kinase can be activated by both PKC-dependent and tyrosine kinase-dependent pathways that appear to converge at some point upstream of MAP kinase kinase.

Since AgR-stimulated PIP_2 hydrolysis leads to activation of PKC, activation of MAP kinase by the B cell AgR could be due entirely to PKC. This hypothesis was tested using a relatively specific inhibitor of PKC, Roche compound No. 3 (Davis *et al.*, 1989). Compound 3 concentrations of 1–5 μM inhibited PKC-mediated MAP kinase activation (induced by phorbol dibutyrate) by greater than 90%, but inhibited anti-Ig-stimulated MAP kinase activation by only 10–40% (Gold *et al.*, 1992a). The simplest interpretation of these results is that much of the anti-Ig-induced MAP kinase activation proceeds by a PKC-independent pathway that is probably initiated by tyrosine kinase activation. One interesting possibility is that PKC ζ may be capable of activating MAP kinase, in which case activation of this PKC isoform by the PI 3-kinase pathway would play a role in activating MAP kinase in B cells.

MAP kinase activation is likely to be an essential component of signal transduction by the B cell AgR. The MAP kinases have multiple roles in receptor-mediated signaling, including phosphorylating and activating the S6 ribosomal protein kinase $p90^{rsk}$ (Sturgill, 1988), which is thought to regulate protein synthesis. In addition, MAP kinases may link cytoplasmic signaling events with nuclear events that regulate gene expression. For example, MAP kinase phosphorylates both c-*myc* (Alvarez *et al.*, 1991) and c-*jun* (Pulverer *et al.*, 1991). c-Jun is a component of the AP-1 transcription factor that regulates gene expression in response to PKC activation. AgR signaling has been shown to stimulate the transcription of c-*myc*, c-*fos*, c-*jun*, *egr-1*, and several other genes in a PKC-dependent manner (Klemsz *et al.*, 1989; Seyfert *et al.*, 1990; Mittelstadt and DeFranco, 1993) (see later).

6. *CD22*

As mentioned previously, an alternative approach to identifying tyrosine kinase substrates is to purify tyrosine-phosphorylated proteins by affinity chromatography and then produce antibodies against the proteins and/or analyze them by protein microsequencing. Schulte *et al.* (1992) made monoclonal antibodies against tyrosine-

phosphorylated proteins isolated from anti-Ig-stimulated Ramos cells (a human B cell lymphoma). One monoclonal antibody recognized a 140-kDa protein. Purification of this protein, followed by sequencing of tryptic peptides derived from it, revealed that it was the CD22 protein. Immunoprecipitation with anti-CD22 antibodies followed by anti-P-tyr immunoblotting confirmed that CD22 was tyrosine phosphorylated in response to AgR cross-linking (Schulte *et al.*, 1992; Leprince *et al.*, 1993).

CD22 functions as an adhesion molecule and may mediate the binding of B cells to monocytes, T cells, and other B cells (Stamenkovic and Seed, 1990; Stamenkovic *et al.*, 1991). Phosphorylation of various adhesion molecules has been reported to alter their affinity (Dustin and Springer, 1991; Damsky and Werb, 1992). Since B cells can recognize antigens that are immobilized on the surface of follicular dendritic cells, antigen nonspecific adhesion mediated by CD22 may facilitate antigen recognition. This would be analogous to the role played by CD2 in T cells. CD22 may also participate in signal transduction by the B cell AgR. Antibodies to CD22 enhance the ability of anti-Ig to increase cytosolic calcium and stimulate proliferation (Pezzutto *et al.*, 1988). Moreover, some CD22 can be detected associated with the B cell AgR (Leprince *et al.*, 1993). Consistent with a role for CD22 in AgR signaling is the observation that $CD22^+$ tonsillar B cells exhibit increased cystolic calcium and proliferate in response to anti-Ig, whereas mIg^+, $CD22^-$ B cells do not (Pezzutto *et al.*, 1988). Also interesting in this regard is that the cytosolic domain of CD22 contains an antigen receptor homology motif (see below) (Leprince *et al.*, 1993).

7. *Components of the B cell AgR: Ig-α and Ig-β*

As alluded to previously, ligand binding to tyrosine kinase receptors such as the PDGF-R leads to tyrosine phosphorylation of the cytoplasmic domain of the receptor at multiple sites (Cantley *et al.*, 1991). Tyrosine phosphorylation of receptors can have important consequences for signal transduction. In particular, it creates specific binding sites for proteins that contain SH2 domains. PLC-γl, *ras*GAP, and PI 3-kinase each bind to a different phosphotyrosine-containing region of the PDGF-R cytoplasmic domain (Fantl *et al.*, 1992). This presumably facilitates the tyrosine phosphorylation of these proteins by the kinase activity of the PDGF-R. It also concentrates important signal transduction proteins in close proximity to each other and to the plasma membrane. Signal transduction may therefore be made more efficient by bringing enzymes such as PLC and PI 3-kinase in contact with their substrates and by bringing the various components of signal-

ing pathways in close physical proximity to each other. Immunofluorescence with anti-P-tyr antibodies reveals that cross-linking of the B cell AgR causes an accumulation of tyrosine-phosphorylated proteins underneath the capped AgR (Takagi *et al.*, 1991). This suggests that similar signaling complexes may be formed in B cells.

AgR cross-linking induces the rapid tyrosine phosphorylation of Ig-α, and to a lesser extent Ig-β (Gold *et al.*, 1991). Cross-linking either mIgM- or mIgD-containing AgRs in murine splenic B cells leads to tyrosine phosphorylation only of the Ig-α proteins associated with the cross-linked receptor (Gold *et al.*, 1991). The Ig-α molecules associated with mIgM and with mIgD in splenic B cells differ in molecular mass, allowing them to be distinguished. This selective tyrosine phosphorylation is consistent with a model in which the activated tyrosine kinases remain physically associated with the cross-linked AgR, are not freely diffusible, and are therefore unable to phosphorylate the Ig-α molecules associated with non-cross-linked receptors. The observation that tyrosine phosphorylation is concentrated in the region of the cross-linked receptors supports this view (Takagi *et al.*, 1991). Alternatively, it is possible that AgR cross-linking induces conformational changes in the cytoplasmic domains of the Ig-α and Ig-β proteins that expose sites of phosphorylation. This latter model would not require stable association of activated tyrosine kinases with the cross-linked AgR. Release of activated tyrosine kinases from cross-linked AgRs could allow a single receptor to go through several rounds of activating tyrosine kinases.

The consequences of tyrosine phosphorylation of the B cell AgR are not known. One hypothesis is that it directs SH2-containing proteins such as *ras*GAP, PLC, and PI 3-kinase to the AgR-associated tyrosine kinases and facilitates their tyrosine phosphorylation. This would be analogous to the function fulfilled by tyrosine phosphorylation of the PDGF-R. To date, direct evidence for binding of such targets to the Ig-α : Ig-β complex is lacking. Tyrosine phosphorylation could also regulate the interaction of tyrosine kinases with the B cell AgR. Since both the *src*-family tyrosine kinases and *syk* contain SH2 domains, AgR tyrosine phosphorylation may serve to recruit additional tyrosine kinases to the AgR. The SH2 domains for the *src*-family tyrosine kinases bind to a sequence motif in which leucine or isoleucine occupies the position three residues C-terminal to the phosphotyrosine (Waksman *et al.*, 1993; Eck *et al.*, 1993; Songyang *et al.*, 1993). As shown in Fig. 2, this sequence is present in both Ig-α and Ig-β and is part of the antigen receptor homology I motif (see later).

Another proposed function for tyrosine phosphorylation of the B cell

Consensus	$^{D}_{E}$xx Yxx$^{L}_{I}$ xxxxxxx Yxx$^{L}_{I}$
Ig-α	ENL YEGL NLDDCSM YEDI
Ig-β	DHT YEGL NIDQTAT YEDI
TCR-ζ1	NQL YNEL NLGRREE YDVL
TCR-ζ2	EGV YNAL QKDKMAEAYSEI
TCR-ζ3	DGL YQGL STATKDT YDAL
CD3-γ	EQL YQPL KDREYDQ YSHL
CD3-δ	EQL YQPL RDREDTQ YSRL
CD3-ε	NPD YEPI RKGQRDL YSGL
FcεRI-β	DRL YEEL NVYSPI YSEL
FcεRI-γ	DAV YTGL NTRSQET YETL

FIG. 2. The antigen receptor homology 1 motif. Murine sequences comprising this motif from the B cell antigen receptor, the T cell antigen receptor, and the high-affinity receptor for IgE of mast cells are shown. Sequences are from Wegener *et al.* (1992) and Reth (1992).

AgR is that it targets the receptor for internalization. Tyrosine kinase inhibitors block internalization of cross-linked AgRs on murine splenic B cells (Puré and Tardelli, 1992). Although these experiments say only that tyrosine phosphorylation of some component is required for AgR internalization, it is tempting to speculate that the relevant component is AgR. If tyrosine phosphorylation does enhance AgR internalization, then it could contribute to the antigen-presenting function of B cells.

In summary, a number of targets of AgR signaling have been identified, although our knowledge of how they contribute to the ultimate biological responses induced by this receptor is still fragmentary. Many of these receptor signaling events and their interconnections are shown in Fig. 3.

D. How Does the B Cell AgR Activate Tyrosine Kinases?

1. Dissecting the AgR

The mIgs have very short cytoplasmic domains (3 amino acids in the case of mIgM and mIgD) whereas the cytoplasmic tails of Ig-α and Ig-β are 61 and 48 amino acids, respectively. Therefore, it is likely that Ig-α and Ig-β are the components of the B cell AgR that interact with cytoplasmic signal transduction molecules such as tyrosine kinases. Consistent with this model, when splenic B cells are solubilized under conditions that disrupt the noncovalent association of mIg with the Ig-α : Ig-β heterodimer, the AgR-associated tyrosine kinases remain associated with the Ig-α : Ig-β complex and not with the mIg (Lin and

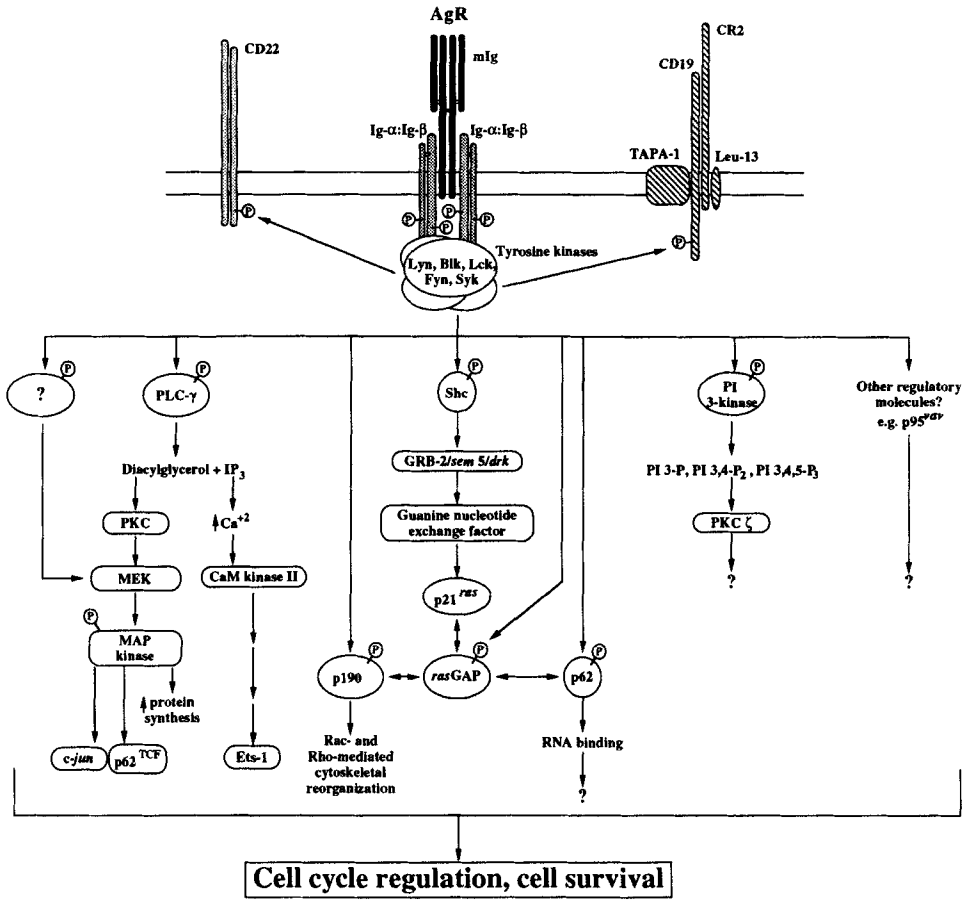

FIG. 3. Signal transduction events initiated by the B cell antigen receptor (AgR). Activation of multiple tyrosine kinases is thought to be the initial signal transduction event stimulated by AgR cross-linking. Arrows leading directly from the tyrosine kinases indicate tyrosine phosphorylation (P) events that are direct or indirect consequences of the activation of Lyn, Blk, Fyn, Lck, and PTK72/*syk*. In no case is it known which tyrosine kinase is responsible for phosphorylating a particular substrate. Arrows that are downstream of the targets of AgR-induced tyrosine phosphorylation represent other signaling reactions (e.g., protein/protein interactions, serine/threonine phosphorylation, and signaling events induced by other second messengers, such as Ca^{2+}). The unidentified tyrosine kinase substrate indicated to the far left represents an unknown intermediate that is involved in PKC-independent activation of MAP kinase. Since MEK is activated by serine/threonine phosphorylation, PKC-independent MAP kinase activation likely involves activation of a serine/threonine protein kinase that regulates MEK. Abbreviations: mIg, membrane immunoglobulin; PLC-γ, phospholipase C γ1 and γ2; PKC, protein kinase C; MEK, MAP kinase/ERK kinase (also known as MAP kinase kinase); IP_3, inositol, 1,4,5-trisphophate; CaM kinase II, Ca^{2+}/calmodulin-dependent protein kinase II; *ras*GAP, $p21^{ras}$ GTPase-activating protein; PI 3-kinase, phosphatidylinositol 3-kinase; PI 3-P, PI 3,4-P_2, and PI 3,4,5-P_3, phosphatidylinositol 3-phosphate, 3,4-bisphosphate, and 3,4,5-trisphosphate, respectively.

Justement, 1992). These experiments do not, however, prove that Ig-α and/or Ig-β interact directly with the tyrosine kinases. It is possible that the kinases interact with a yet unidentified component of the AgR that can form a stable association with the Ig-α : Ig-β heterodimer. One way to distinguish these possibilities is to express the B cell AgR in other cell types that presumably do not express B cell-specific molecules. Matsuuchi *et al.* (1992) have expressed the B cell AgR in an endocrine cell line, AtT20, by transfecting the cells with genes encoding μ, λ, Ig-α, and Ig-β. Cross-linking of the transfected AgR in these cells induces tyrosine phosphorylation of Ig-α and Ig-β that is as strong as it is in B cells. This observation suggests that the tyrosine kinases that mediate these responses can interact directly with Ig-α and/or Ig-β, since it is unlikely that AtT20 cells express other possible components of the B cell AgR. It should be noted, however, that although anti-Ig stimulation of the transfected AtT20 cells induces substantial tyrosine phosphorylation of Ig-α and Ig-β, other signaling events are weak (tyrosine phosphorylation of PI 3-kinase) or undetectable (PIP_2 breakdown). Thus, a lymphoid-specific component appears to be required for full AgR signaling.

Since the B cell AgR activates multiple tyrosine kinases and may also interact with other signaling molecules, including substrates for these kinases, the question arises as to whether different regions of the Ig-α and Ig-β cytoplasmic domains mediate distinct functions. An 18-amino acid motif (D/E-x-x-Y-x-xL/I-x-x-x-x-x-x-Y-x-xL/I, where x is any amino acid) that is found in the cytoplasmic domains of the CD3 γ, δ, ε, and ζ components of the T cell AgR, in the β and γ subunits of the mast cell IgE receptor (FcεRI), and in the human FcγRIIA is also present in both Ig-α and Ig-β (Fig. 2) (Reth, 1989). Since these components are thought to mediate signaling by their respective receptors, it has been postulated that this motif, termed the antigen receptor homology I motif (ARH1) (Cambier, 1992) or tyrosine-based activation motif (TAM) (Samelson and Klausner, 1992), constitutes a "signaling domain." Chimeric proteins in which the cytoplasmic domain consists of only a single ARH1 domain from either the ζ chain or CD3ε have been expressed in T cells and shown to be able to mediate many of the signaling reactions characteristic of the intact T cell receptor, including tyrosine phosphorylation, calcium mobilization, stimulation of IL-2 production, and induction of cytolytic activity (Romeo *et al.*, 1992; Letourneur and Klausner, 1992; Irving *et al.*, 1993). The two tyrosines in the CD3ε and ζ chain ARH1 domains are essential for signaling function of chimeras containing these motifs (Romeo *et al.*, 1992; Letourneur and Klausner, 1992). Among ARH1 domains, the noncon-

served amino acids can diverge extensively, suggesting that different ARH1 domains could mediate different signaling functions. Indeed, fusion proteins containing the ARH1 domains from the ζ chain and CD3ε appear to stimulate different patterns of tyrosine phosphorylation on cross-linking (Letourneur and Klausner, 1992) and may also differ in their ability to stimulate other responses (Wegener *et al.*, 1992).

Ig-α and Ig-β each contain one ARH1 motif. The ARH1 motifs in Ig-α and Ig-β differ at 8 of 18 amino acid residues, raising the possibility that they could mediate different signaling reactions. M. R. Clark *et al.* (1992) have attempted to separate the functions of Ig-α and Ig-β by looking for proteins that bind *in vitro* to the cytoplasmic domains of Ig-α and Ig-β. Glutathione-S-transferase fusion proteins containing the the entire cytoplasmic domain of Ig-α or Ig-β were immobilized on glutathione–Sepharose beads, mixed with B cell lysates, and proteins that remained bound to the fusion proteins after extensive washing were detected by the addition of labeled ATP. Since the fusion proteins have no kinase activity, the underlying assumption is that kinases will bind to the fusion proteins and then phosphorylate other proteins that bind. Although no kinases bind to glutathione-S-transferase alone, kinases that phosphorylate the AgR components bind to both Ig-α and Ig-β fusion proteins. The Ig-α protein is phosphorylated on serine, threonine, and tyrosine residues, indicating that both tyrosine and serine/threonine kinases bind to the cytoplasmic domain of Ig-α. In contrast, Ig-β is phosphorylated primarily on serine and threonine residues with only a small amount of tyrosine phosphorylation. Elution of proteins from the fusion protein beads followed by immunoprecipitation with antibodies against tyrosine kinases shows that $p59^{fyn}$ and $p53/p56^{lyn}$ bound preferentially to Ig-α, although the efficiency of this interaction was not assessed. In addition, these *in vitro* kinase assays detect proteins of 76 and 85 kDa that bind to both the Ig-α and the Ig-β beads, several proteins of 50–59 kDa (likely to be tyrosine kinases), a 38-kDa protein that bind exclusively to the Ig-α beads, and two proteins of 40 and 42 kDa (which are not MAP kinases) that bind exclusively to the Ig-β beads. Furthermore, immunoblotting showed that PI 3-kinase can bind to both Ig-α and Ig-β beads. Although it is not clear whether these proteins bind directly to the Ig-α and Ig-β cytoplasmic tails or to the kinases that bind to them, these results suggest that different functions are carried out by the two invariant chains of the B cell AgR. Moreover, similar results are obtained using fusion proteins that contain only a 26-amino acid region encompassing the ARH1 domain, arguing that these regions contain

the necessary structural information required for Ig-α and Ig-β to interact with kinases and other signaling components.

Although these results are intriguing, one possible shortcoming of this approach is that it is difficult to judge the extent to which these proteins interact with each other and whether those levels are physiologically significant. The assay combines large amounts of cell lysate and fusion protein and binding is then detected by an extremely sensitive assay. Therefore, these results will need to be confirmed by *in vivo* experiments in which the isolated cytoplasmic domains of Ig-α and Ig-β are expressed in B cells as fusions with other membrane proteins. Law *et al.* (1993) have made fusion proteins containing the external domain of μ, and the transmembrane and cytoplasmic domains of CD8 fused to 30 amino acid regions containing the ARH1 of either Ig-α or Ig-β. These fusion proteins were expressed in the IgG-bearing B cell line 2PK-3 and then assayed for their ability to stimulate signaling events characteristic of the B cell AgR when cross-linked with anti-IgM antibodies. In contrast to the *in vitro* results of M. R. Clark *et al.* (1992), which suggested different functions for Ig-α and Ig-β, Law *et al.* (1993) found that both the Ig-α and the Ig-β fusion proteins are able to reproduce the signaling events stimulated by the intact AgR when expressed in 2PK-3 cells. Cross-linking of either fusion protein induces the same pattern of tyrosine phosphorylation as does the IgG-containing AgR in these cells. In addition, both Ig-α and Ig-β fusion proteins stimulate PIP_2 hydrolysis as well as tyrosine phosphorylation of $p95^{vav}$ and both subunits of PI 3-kinase. All of these signaling properties required the added portions of Ig-α or Ig-β. Chimeras lacking these regions are completely unable to signal. Both the Ig-α chimera and the Ig-β chimera are tyrosine phosphorylated on cross-linking. Based on these results, it would seem that Ig-α and Ig-β are redundant in terms of many signal transduction functions, although both are clearly required for transport of the mIg-containing AgR to the cell surface (Hombach *et al.*, 1990b; Venkitaraman *et al.*, 1991; Matsuuchi *et al.*, 1992).

Site-directed mutagenesis of the Ig-α and Ig-β ARH1 domains should further define the critical residues required for activation of tyrosine kinases by the B cell AgR. Of particular interest are the two tyrosine residues in the ARH1 motifs. Changing either of the two consensus tyrosines to phenylalanines in the CD3ε or ζ ARH1 domains severely inhibits the ability of fusion proteins containing these domains to transmit signals in T cells (Romeo *et al.*, 1992; Letourneur and Klausner, 1992). Because phenylalanine is very similar in structure to

tyrosine, but cannot be phosphorylated, this result suggests that tyrosine phosphorylation of the receptor is essential for signal transduction. One possibility is that the ARH1 motif is actually two SH2 binding sites. In this context, it should be noted that both halves of the ARH1 motif are good binding sites for *src*-family tyrosine kinases (Songyang *et al.*, 1993). Moreover, PTK72/*syk* and ZAP70 have SH2 domains that resemble *src*-family SH2 domains in sequence and may have similar specificity for binding (Songyang *et al.*, 1993). Indeed, Law *et al.* have found that stimulation of chimeras containing Ig-α or Ig-β leads to increased binding of PTK72/*syk*, p53/p56^{lyn} and p59^{fyn} (Law *et al.*, 1993). This increased binding of tyrosine kinases to the cytoplasmic domains of the AgR may play an important role in signal initiation (see later).

2. *Regulation of the src Family of Tyrosine Kinases*

Activation of *src*-family tyrosine kinases is controlled, at least in part, by phosphorylation at a tyrosine residue near the carboxy terminus that is conserved in all eight known members of this family. Phosphorylation at this site (Y505 in p56^{lck}, Y527 in p60^{src}) inhibits kinase activity (Amrein and Sefton, 1988; Marth, 1988; Okada *et al.*, 1991; Bergman *et al.*, 1992). In contrast, changing this tyrosine residue to phenylalanine, which cannot be phosphorylated, results in constitutively activated kinases that are oncogenic in fibroblasts (Cartwright *et al.*, 1987; Amrein and Sefton, 1988; Hunter, 1987). When phosphorylated at the negative regulatory site, the carboxy-terminal tail of the *src*-like kinases may bind the SH2 domain of the kinase in an intramolecular interaction that keeps the kinase in an inactive conformation (Cantley *et al.*, 1991; Roussel *et al.*, 1991; Sieh *et al.*, 1993). The level of phosphorylation at the negative regulatory site in the *src*-family kinases appears to reflect a dynamic equilibrium between opposing kinases and phosphatases. Among the prime candidates for these regulators of the *src*-family kinases are the tyrosine phosphatase CD45 and a tyrosine kinase called p50^{csk} (for c-*src* kinase).

p50^{csk} is a 50-kDa ubiquitously expressed tyrosine kinase that can phosphorylate p60^{src}, p56^{lck}, p56^{lyn}, and p59^{fyn} (Bergman *et al.*, 1992; Okada *et al.*, 1991) *in vitro* at the negative regulatory site and inhibit the activity of these kinases. Because the other *src*-family tyrosine kinases, such as p55^{blk}, also have the conserved negative regulatory site, it is likely that p50^{csk} can phosphorylate and inhibit the activity of these kinases as well. Thus, p50^{csk} could simultaneously regulate the activity of all of the *src*-family tyrosine kinases that are activated by the

B cell AgR. At this point, however, nothing is known about how $p50^{csk}$ is regulated or whether it regulates the *src*-family tyrosine kinases in a dynamic fashion during AgR signaling.

Since phosphorylation of *src*-family kinases at the negative regulatory site inhibits their activity, tyrosine phosphatases may play an essential role in the activation of these kinases. CD45 is a transmembrane tyrosine phosphatase that is expressed at very high levels on all hematopoietic cells. There is considerable evidence in T cells that CD45 regulates the activity of *src*-family tyrosine kinases, particularly $p56^{lck}$ and $p59^{fyn}$. Mutant T cell lines lacking CD45 show greatly decreased protein tyrosine phosphorylation, calcium elevation, and IL-2 production in response to T cell receptor (TCR) cross-linking (Pingel and Thomas, 1989; Koretzky *et al.*, 1991; Shiroo *et al.*, 1992), and expression of CD45 in these cells restores normal responses (Koretzky *et al.*, 1991). Regulation of these kinases by CD45 most likely involves controlling the level of phosphorylation at the negative regulatory sites of these kinases. In T cell lines lacking CD45 there is increased phosphorylation of $p56^{lck}$ at the negative regulatory site (Ostergaard *et al.*, 1989). $p59^{fyn}$ activity is also greatly reduced in CD45-negative cells (Shiroo *et al.*, 1992). Correspondingly, incubation with CD45 can activate $p56^{lck}$ and $p59^{fyn}$ *in vitro* (Mustelin *et al.*, 1989, 1992). These results suggest the possibility that the initial event in T cell receptor-induced tyrosine kinase activation is dephosphorylation of TCR- and/or CD4-associated *src*-family tyrosine kinases by CD45. It is not clear how CD45 activity is regulated. An interaction between the TCR and CD45 has been detected (Volarevic *et al.*, 1990) and this interaction may be altered by ligand-induced TCR clustering. Interestingly, cross-linking of the TCR induces transient tyrosine phosphorylation of CD45, suggesting some sort of regulation (Stover *et al.*, 1991). In addition, elevation of intracellular free calcium causes an increase in the phosphatase activity of CD45 in T cells (Ostergaard and Trowbridge, 1991).

A role for CD45 in the activation of tyrosine kinases by the B cell AgR has also been suggested. Justement *et al.* (1991) transfected the gene encoding Ig-α into the CD45-negative plasmacytoma cell line J558 to allow expression of the B cell AgR on the cell surface. Cross-linking of the AgR in this cell line fails to induce an increase in intracellular calcium, but this response can be restored by expression of CD45 (Justement *et al.*, 1991). Experiments by Reth and colleagues have shown that, in contrast to T cells, CD45 is not required for induction of protein tyrosine phosphorylation by anti-Ig, but rather is only required for activation of phospholipase C (Kim *et al.*, 1993).

Similarly, cross-linking CD45 with anti-CD45 monoclonal antibodies (mAbs) inhibits anti-Ig-induced calcium increases, but does not block anti-Ig-induced protein tyrosine phosphorylation (Lane *et al.*, 1991). Cross-linking CD45 with mAbs could activate its phosphatase activity, or alternatively sequester it away from the AgR and thus block its ability to participate in AgR signaling. As in T cells, there is some evidence for a physical association of CD45 with mIg. Overnight incubation of splenic B cells with anti-CD45 mAb down-regulated both CD45 and mIg on about 35% of the cells (Justement *et al.*, 1991). This result has been interpreted to reflect association of CD45 with the AgR complex, although other possibilities can be entertained.

Much less is known about how the PTK72/*syk* tyrosine kinase is regulated. The *syk* gene product lacks the consensus *src*-family negative regulatory site (Taniguchi *et al.*, 1991). Thus, PTK72/*syk* is probably regulated in a very different manner than are the *src*-family tyrosine kinases.

3. *Functions of the Different Tyrosine Kinases Associated with the B Cell AgR*

Why does the B cell AgR associate with at least five different tyrosine kinases? Does each kinase have a distinct function or are they redundant? The notion that the functions of the different *src*-family tyrosine kinases may be redundant was prompted by experiments in which transgenic mice lacking $p60^{src}$ were produced (Soriano *et al.*, 1991). Although $p60^{src}$ is highly expressed in the nervous system and in platelets, $p60^{src}$ knockout mice are essentially normal except for a bone defect. This observation argues that the functions of $p60^{src}$ in the nervous system and elsewhere can be performed by another kinase, presumably another member of the *src* family. Other experiments suggest that not all of the *src*-like kinases can perform the same functions. For example, knockout mutations of *fyn* and *lck* have different effects on T cell development and signaling (Appleby *et al.*, 1992; Stein *et al.*, 1992; Molina *et al.*, 1992). One argument supporting the possibility of redundancy of the AgR-associated tyrosine kinases in B cells is that different cell lines exhibit very similar patterns of anti-Ig-induced tyrosine phosphorylation on anti-P-tyr immunoblots, despite expressing somewhat different *src*-family tyrosine kinases. For example, the WEHI-231 and BAL 17 B cell lines show very similar patterns of tyrosine phosphorylation (Gold *et al.*, 1991), although WEHI-231 expresses $p56^{lck}$ but not $p59^{fyn}$ and BAL 17 expresses $p59^{fyn}$ but not $p56^{lck}$ (*Law et al.*, 1992; I. van Oostveen and M. Gold, unpublished observations). In contrast, $p55^{blk}$ and $p53/p56^{lyn}$ are ubiquitously ex-

pressed in different B cell lines (Dymecki *et al.*, 1990; Law *et al.*, 1992). It seems likely that some functions of the different AgR-associated tyrosine kinases overlap whereas others are unique. In particular, the PTK72/*syk* kinase, which is unrelated to the *src*-family tyrosine kinases (Taniguchi *et al.*, 1991), would be expected to have unique functions.

4. A Model of AgR Signal Initiation

In resting B cells, only a small fraction of the AgRs appear to be associated with tyrosine kinases (Sefton and Campbell, 1991; Lin and Justement, 1992). As cross-linking AgRs induces increased tyrosine phosphorylation of Ig-α and Ig-β (Gold *et al.*, 1991), and as it also leads to increased binding of p53/56^{lyn}, p59^{fyn}, and PTK72/*syk* to the cytoplasmic tails of these proteins (Law *et al.*, 1993), one attractive hypothesis is that these kinases are bound to the AgR via their SH2 domains interacting with tyrosine-phosphorylated ARH1 motifs of Ig-α and Ig-β. The SH2 domains of p56^{lck} and p60^{src} preferentially bind to the sequence motif phosphoY-x-x-L (Eck *et al.*, 1993; Waksman *et al.*, 1993; Songyang *et al.*, 1993), which is found in the ARH1 domains of both Ig-α and Ig-β. The SH2 domains of the other *src*-family tyrosine kinases appear to have similar specificity (Songyang *et al.*, 1993). PTK72/*syk* contains two SH2 domains. Although the binding specificities of these SH2 domains have not been determined experimentally, analysis of the sequences suggests that these SH2 domains have specificities similar to those of SH2 domains of the *src*-family kinases (Songyang *et al.*, 1993). Moreover, experiments with chimeric proteins containing ARH1 domains from the T cell receptor support the view that phosphorylation of the tyrosine residues in the ARH1 motif is involved in the interaction with tyrosine kinases. Changing the tyrosine residues in the ARH1 domains to phenylalanine residues, which are structurally similar to tyrosine but cannot be phosphorylated, greatly decreases the signaling ability of these chimeric proteins (Romeo *et al.*, 1992; Letourneur and Klausner 1992; Irving *et al.*, 1993).

Thus, the low amount of tyrosine kinase bound to the AgR prior to stimulation could be due to binding to the small amount of tyrosine-phosphorylated Ig-α and Ig-β found in the unstimulated cell. In any case, cross-linking AgRs would bring a few AgR-associated tyrosine kinases into contact with many more AgR cytoplasmic tails. The clustering of substrates next to tyrosine kinases would lead to rapid tyrosine phosphorylation of some of the Ig-α and Ig-β molecules. This would be followed by binding of p53/56^{lyn}, p59^{fyn}, and PTK72/*syk* to these newly phosphorylated tails and subsequent phosphorylation of

additional Ig-α/Ig-β tails and other substrates. The dependence of AgR phosphorylation on cross-linking has been demonstrated in splenic B cells in which cross-linking of either mIgM- or mIgD-containing AgRs leads to increased tyrosine phosphorylation only of the Ig-α molecules associated with the type of AgR that has been cross-linked (Gold *et al.*, 1991). Moreover, cross-linking of chimeric proteins containing the Ig-α or the Ig-β ARH1 motifs in a B cell line has been shown to induce tyrosine phosphorylation of these proteins and to increase the amount of PTK72/*syk*, p53/p56^{lyn}, and p59^{fyn} associated with these chimeric proteins (Law *et al.*, 1993). Similarly, increased association of the ZAP70 tyrosine kinase, the T cell homologue of PTK72/*syk*, with the TCR is observed after cross-linking with anti-CD3 antibodies (Chan *et al.*, 1991, 1992). Additional studies will be needed to test other aspects of this model, such as the importance of phosphorylation of the ARH1 motif tyrosines and the importance of SH2 domains of the tyrosine kinases for binding to stimulated receptors.

In addition to recruiting additional tyrosine kinases to the AgR, AgR cross-linking may also increase the activity of the kinases that are bound. Several modes of activation may come into play. The SH2-mediated binding of the tyrosine kinases to the phosphoY-x-x-L motifs on the AgR could induce a conformational change that activates the tyrosine kinase. For example, the binding of a tyrosine-phosphorylated peptide from the IRS-1 protein to the SH2 domain of PI 3-kinase has been shown to increase the activity of PI 3-kinase *in vitro* (Backer *et al.*, 1992). A well-established alternative mechanism of activation for *src*-family tyrosine kinases is dephosphorylation of the negative regulatory site, as discussed earlier. In T cells, the CD45 tyrosine phosphatase is required for efficient dephosphorylation of p56^{lck} (Ostergaard *et al.*, 1989) and p59^{fyn} (Shiroo *et al.*, 1992). CD45 is also required for TCR signaling (Koretzky *et al.*, 1990, 1991), perhaps because failure to dephosphorylate the negative regulatory tyrosine of one or more of the *src*-family kinases inhibits TCR signaling. Similarly in B cells, AgR cross-linking may induce CD45-mediated dephosphorylation of the *src*-family tyrosine kinases associated with the B cell AgR. There is some evidence that the B cell AgR physically associates with CD45 (Justement *et al.*, 1991). The recruitment of additional tyrosine kinases to the AgR upon cross-linking would bring them in proximity to CD45 and allow them to be dephosphorylated and activated.

The PTK72/*syk* tyrosine kinase does not have the conserved negative regulatory site that is found in the *src*-family tyrosine kinases and must therefore be regulated in another manner. One possibility is that PTK72/*syk* is activated due to phosphorylation by another tyrosine

kinase (e.g., a *src*-family tyrosine kinase). Indeed, anti-Ig treatment leads to increased tyrosine phosphorylation and activation of PTK72/*syk* (Hutchcroft *et al.*, 1991, 1992). The possibility that PTK72/*syk* is activated by tyrosine phosphorylation is supported by experiments in which cells are transfected with $p56^{lck}$, $p59^{fyn}$ or ZAP70 individually or in combinations. Expression of a single tyrosine kinase in COS cells leads to very little tyrosine phosphorylation, whereas coexpression of ZAP70 with either $p56^{lck}$ or $p59^{fyn}$ results in a large increase in the tyrosine phosphorylation of many cellular proteins (Chan *et al.*, 1992). These observations suggest that phosphorylation of ZAP70 by $p56^{lck}$ or $p59^{fyn}$ (and/or vice versa) greatly amplifies the activity of one or both of these kinases. In B cells, a similar interaction may occur between PTK72/*syk* and the *src*-family kinases associated with the AgR. In unstimulated cells, very few AgRs have associated tyrosine kinases and the chance that a single AgR has more than one associated kinase is low. AgR cross-linking would recruit more tyrosine kinases to the AgRs and bring them in close proximity to each other, favoring an interaction between the two classes of kinases (Fig.4).

Tyrosine phosphorylation of the AgR and the associated tyrosine kinases could also attract SH2-containing signaling targets for the AgR-associated tyrosine kinases, thereby facilitating their phosphorylation. Among the known tyrosine kinase substrates in B cells, PLC-γ, *ras*GAP, PI 3-kinase, and $p95^{vav}$ all contain SH2 domains. Whether the SH2 domains of these proteins are needed for them to be tyrosine phosphorylated in B cells is not known. In addition to facilitating their tyrosine phosphorylation, SH2-mediated binding of PLC-γ *ras*GAP, and PI 3-kinase to the AgR complex or to associated proteins such as CD19 or CD22 would bring these cytoplasmic proteins in close proximity to their substrates, which reside in the plasma membrane. In fibroblasts, PLC-γ, *ras*GAP, and PI 3-kinase form a complex with PDGF-R (Cantley *et al.*, 1991). It is not clear whether the AgR nucleates an analogous signaling complex in B cells. Another possibility is that there is a protein that serves as a link between the AgR complex with its associated tyrosine kinases and the signaling component targets, analogous to the role played by the IRS-1 protein in insulin receptor signaling (Sun *et al.*, 1991).

After several minutes of this amplification process, negative-feedback loops are activated, serving to return the system to its basal state. Down-regulation of tyrosine kinase activation and protein tyrosine phosphorylation could proceed by several mechanisms. $p50^{csk}$ contains an SH2 domain (Nada *et al.*, 1991) and may bind to autophosphorylated *src*-family tyrosine kinases, phosphorylate them at the neg-

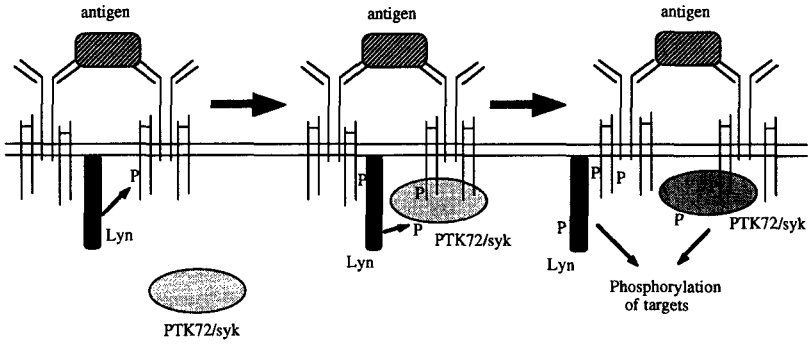

FIG. 4. Model for initiation of signal transduction on antigen binding. Multivalent or oligovalent antigen binding to the AgR cross-links several AgR molecules and brings the ARH1 motifs into contact with a few *src*-family tyrosine kinases ($p53/56^{lyn}$ is shown), which are either bound to AgRs in low stoichiometry or are trapped by the cross-linking (left). These *src*-family tyrosine kinases phosphorylate the ARH1 motifs, after which PTK72/*syk* and $p53/56^{lyn}$ and $p59^{fyn}$ can bind via their SH2 domains (middle). The bound tyrosine kinase molecules may now be substrates for phosphorylation as well, and this phosphorylation may activate the bound tyrosine kinase molecules. In any case, the assembly of tyrosine kinases on the cytoplasmic tails of the AgR Ig-α and Ig-β components allows them to phosphorylate targets, including important signal-transducing components (right). It is not known whether signaling targets are phosphorylated by PTK72/*syk* or by *src*-family tyrosine kinases or by both.

ative regulatory site, and decrease their activity. Phosphorylation of *src* kinases at other sites may also down-regulate their activity. In both T and B cells, AgR signaling induces phosphorylation of $p56^{lck}$ at serine 59 (J. D. Watts *et al.*, 1993; Gold *et al.*, 1993c). Phosphorylation of $p56^{lck}$ at this site *in vitro* inhibits its enzymatic activity (J. D. Watts *et al.*, 1993). Tyrosine phosphatases, including CD45, may also play a role in down-regulating kinase activity and in decreasing the levels of protein tyrosine phosphorylation. CD45 could dephosphorylate the AgR as well as the substrates of the AgR-associated tyrosine kinases. Justement *et al.* (1991) have shown that Ig-α and Ig-β are both substrates for CD45 *in vitro*. Dephosphorylation of the AgRs could decrease the association of tyrosine kinases with the AgR and reduce signaling. Finally, tyrosine-phosphorylated AgRs could be targeted for internalization, which might halt signaling reactions by removing the AgR from necessary signaling components in the plasma membrane.

5. *Coreceptors for the B Cell AgR?*

In T cells, the CD4 and CD8 molecules play an important role in T cell activation through the TCR. CD4 and CD8 bind to class II and

class I MHC molecules, respectively, on the antigen-presenting cell. However, they do not merely serve to increase the affinity of the T cell/antigen-presenting cell interaction, since efficient T cell activation requires that the CD4 or CD8 molecule and the TCR bind to the same MHC molecule (Janeway, 1992). This implies that an interaction between the TCR and the CD4 or CD8 "coreceptor" is required for optimal signaling. As the cytoplasmic domains of CD4 and CD8 bind $p56^{lck}$ with high affinity (Rudd *et al.*, 1988; Veillette *et al.*, 1988), an attractive possibility is that contact of the TCR and CD4 or CD8 with the same peptide/MHC complex serves to bring together a tyrosine kinase ($p56^{lck}$) and substrates for it (ARH1 motifs) in the cytoplasm of the T cell. This would be an efficient mechanism for inducing phosphorylation of the ARH1 motifs of CD3γ, δ, ε, and ζ chains, setting in motion the events described previously. Thus, the inclusion of a coreceptor would improve the efficiency of signal initiation compared to simple cross-linking of T cell AgRs with relatively few prebound tyrosine kinases.

Is there an analogous coreceptor for the B cell AgR? The best candidate for such a coreceptor is a membrane protein complex expressed on B cells that includes complement receptor 2(CR2), CD19, TAPA-1, and Leu-13 (Matsumoto *et al.*, 1991; Bradbury *et al.*, 1992; van Noesel *et al.*, 1993). CR2, also known CD21, is the receptor for C3dg and iC3b complement fragements (Fearon and Wong, 1983). CD19 has a large extracellular domain, suggesting that it could also serve as a binding component for other, currently unknown ligands. Several observations suggest that this complex could be a coreceptor for the B cell AgR. Although CD19, like the B cell AgR, can stimulate inositol phosphate production and calcium increases, co-cross-linking of CD19 to the B cell AgR results in synergistic responses (Carter *et al.*, 1991b). Synergistic B cell proliferation can also be induced in this way (Carter and Fearon, 1992). In addition, about one-third of the CD19 molecules on the B cell surface can be comodulated by cross-linking of the AgR, indicating a physical association (Pesando *et al.*, 1989). Moreover, some CD19 antibodies inhibit B cell activation via the B cell AgR (Barrett *et al.*, 1990), perhaps by sequestering the CD19/CR2 complex away from the AgR. These phenomena are very similar to those described earlier for CD4 and suggest that the CD19/CR2 complex may have an analogous coreceptor function.

Many real antigens would fix complement by the alternative pathway or by binding low-affinity cross-reactive IgM molecules. Such antigens coated with complement components could co-cross-link the CD19/CR2 complex with the B cell AgR. Consistent with a role for the

CD19/CR2 complex in B cell activation via the AgR, complement-deficient animals have impaired antibody production (reviewed in van Noesel *et al.*, 1993) and soluble forms of CR2 strongly inhibit antibody responses, presumbably by interacting with fragments of C3b on antigens and preventing their interaction with CR2 on B cells (Hebell *et al.*, 1991).

CR2 in not required for assembly of CD19 with TAPA-1, and cross-linking of such CR2-deficient complexes with anti-CD19 antibodies still synergizes with AgR cross-linking (Carter *et al.*, 1991b). This observation suggests that CR2 performs a ligand-binding function while signaling functions are carried out by CD19 or another member of the complex. Thus, binding of a relevant ligand to CD19 or one of the other components of the complex would also be able to invoke the proposed coreceptor function of this complex (van Noesel *et al.*, 1993). Since antigens are often immobilized and concentrated on antigen-presenting cells in the lymphoid organs, one could postulate that CD19 is an adhesion molecule that recognizes some component on the antigen-presenting follicular dendritic cell, much the way that CD4 recognizes MHC class II molecules on antigen-presenting cells. Recently, CR2 was shown to bind the CD23 molecule, which is expressed on many hematopoietic cells (Aubry *et al.*, 1992).

The mechanism by which the CD19 complex enhances signaling by the B cell AgR is not understood. Cross-linking the CD19 complex stimulates tyrosine phosphorylation of only a single protein of 85 kDa (Carter *et al.*, 1991b). This suggests that a tyrosine kinase is associated with the CD19 complex, although perhaps not at the high stoichiometry with which $p56^{lck}$ is associated with CD4. There is preliminary evidence that $p53/56^{lyn}$ is associated with CD19 (van Noesel *et al.*, 1993). Cross-linking CD19 stimulates inositol phosphate production in a tyrosine kinase-dependent fashion, but in contrast to the AgR, this does not involve tyrosine phosphorylation of PLC-γ1 (Carter *et al.*, 1991b). Thus, the CD19 complex activates PLC by a different mechanism than does the AgR. Another interesting observation is that although anti-Ig-induced inositol phosphate production is inhibited by protein kinase C, activation of PLC by anti-CD19 or by anti-CD19 plus anti-Ig is not (Carter *et al.*, 1991b). Thus, a negative-feedback loop exists in which PLC activation leads to activation of PKC and this down-regulates PLC activity. Signaling by the CD19 complex may overcome this inhibition, allowing for sustained signaling by the AgR. Clearly much more needs to be learned about signaling by the CD19 complex and its role in B cell activation through the AgR.

V. Early Cellular Events Following Antigen Contact

A. Cytoskeletal Attachment, Capping, and Antigen Uptake

Soon after anti-Ig or multivalent antigen engages the AgR, it becomes attached to the cytoskeleton. This is manifested by lack of solubility in nonionic detergents such as Triton X-100 or NP-40 (Braun *et al.*, 1982; Woda and Woodin, 1984). These AgR molecules can be solubilized by treatment of the cells with agents that disrupt microfilaments, such as cytochalasins (Rothstein, 1986). The attachment of the AgR to the insoluble cytoskeleton can be followed either by biochemical means or by flow cytometry (Albrecht and Noelle, 1988). Only cross-linked molecules become attached to the cytoskeleton. For example, anti-IgM renders mIgM but not mIgD insoluble. Interestingly, some IgM can be found in an insoluble state prior to anti-Ig cross-linking. This seems to be pentameric secreted IgM, rather than membrane IgM (Albrecht and Noelle, 1989). Presumably this secreted IgM is bound to a transmembrane protein, such as an FcR. Cytoskeletal attachment of mIgM may occur indirectly by anti-IgM-mediated cross-linking of mIgM to already attached secreted IgM (Albrecht and Noelle, 1988). One problem with this interpretation is that there is very little secretory IgD, so the attachment of mIgD would have to proceed by a different mechanism. If this attachment occurs through the Ig-α : Ig-β heterodimer, as seems likely, then mIgM should also be able to attach to the cytoskeleton in this way.

The attachment of the AgR to microfilaments may involve α-actinin (Gupta and Woda, 1988). Prior to ligand interaction with the AgR, some α-actinin and actin can be immunoprecipitated with mIgM and mIgD, but this complex cannot be detected following anti-Ig treatment, presumably because it has become attached to larger cytoskeletal structures. Interestingly, α-actinin also has been found to bind diacylglycerol and palmitic acid (Burn *et al.*, 1985). The former lipid is produced as a result of phosphoinositide breakdown in anti-Ig-treated cells, so AgR signaling could promote α-actinin binding to the membrane and therefore could enhance local interactions of microfilaments with the membrane. Whether or not α-actinin binds directly to the AgR is unclear. It is also possible that other proteins, such as talin or ankyrin, connect microfilaments to the AgR and α-actinin is merely connected via its association with actin filaments.

A number of targets of AgR signaling may be involved in regulating the cytoskeleton. Among these are the *ras*GAP-associated p190, which has GTPase-activating properties for the Rac and Rho subfamily of small-molecular-weight G proteins (Settleman *et al.*, 1992a). These G

proteins have been shown to regulate actin-based cortical microfilaments and actin cables (Ridley *et al.*, 1992; Ridley and Hall, 1992). Also phosphorylated on tyrosine residues following anti-Ig stimulation is $p95^{vav}$ (Bustelo and Barbacid, 1992). This protein has strong homology to *S. cerevisiae* CDC24, which acts as a guanine nucleotide exchange factor for the yeast equivalent of Rho. An equivalent activity for $p95^{vav}$ has not yet been demonstrated. It is tempting to speculate that one or more of the targets of AgR signaling regulate members of the Rac/Rho family, which, in turn, are regulating microfilament assembly. Finally, a major protein kinase C substrate in B cells is the MARCKS protein (Hornbeck *et al.*, 1989). This protein can bind to actin microfilaments in a way that is regulated by intracellular free calcium and protein kinase C (Aderem, 1992). Prior to stimulation of the cell, MARCKS binds to the plasma membrane and also to two actin filaments. Calcium elevation can lead to calmodulin binding in place of one of the actin filaments, whereas phosphorylation by protein kinase C leads to detachment of MARCKS from the membrane. These are provocative findings, and suggest that MARCKS is playing an important role in remodeling cortical actin filaments in cells stimulated through the AgR.

The attachment of AgRs to microfilaments is followed by capping, a process whereby all of the bound AgRs are brought to one pole of the B cell. Capping is an active process; it is blocked by low temperatures, agents that poison the cell's energy supply, and agents that depolymerize microfilaments (Bourguignon and Bourguignon, 1984; DeFranco, 1987). Capping can be triggered by soluble, haptenated protein antigens (oligovalent) as well as by anti-Ig reagents (Snow *et al.*, 1983). In one study involving seven anti-IgM monoclonal antibodies, only three induced cytoskeletal attachment, and the same three antibodies induced capping, whereas the other four did not (Albrecht and Noelle, 1988). This observation and knowledge of the effects of actin-depolymerizing agents strongly suggest that attachment of the AgR to the cytoskeleton is essential to the capping process. Moreover, a variety of proteins of the microfilament cytoskeleton all cocap with the AgR. These include actin, myosin, α-actinin, and fodrin (Bourguignon and Bourguignon, 1984; DeFranco, 1987). A number of signaling components also colocalize with mIg caps. For example, anti-P-tyr antibodies show a concentration of tyrosine-phosphorylated proteins underlying an AgR cap (Takagi *et al.*, 1991). This observation suggests that AgR activation of protein tyrosine kinase(s) is in some way localized to the vicinity of the AgRs. As mentioned above, $p21^{ras}$ also colocalizes with AgR caps (Graziadei *et al.*, 1990), as does calmodulin

(Salisbury *et al.*, 1981). One implication of localized information is that it could be used to promote directional events, such as cell migration.

The biological roles of cytoskeletal attachment and capping are not clear. As discussed later, these events do not seem to be necessary for the uptake of antigen needed for interaction with specific helper T cells. It may be that capping and consequent endocytosis play an important role in AgR down-regulation, and in this way terminate the signaling of AgRs. Further signaling would require expression of newly synthesized AgRs and the continued presence of antigen.

In addition to the endocytosis seen following capping, AgRs also are internalized constitutively in a recycling process. In an Epstein–Barr virus (EBV)-transformed B cell line, 60% of the surface-labeled AgRs are found to be inside the cell at any time (Davidson *et al.*, 1990). Unbound antitetanus toxoid AgRs mainly recycle between early endosomes and the cell surface. AgRs binding a monovalent tetanus toxoid molecule have a higher probability than do free AgRs of going to the proteolytically more active late endosomes, where both antigen and AgR can be degraded. Peptides generated in late endosomes or lysosomes combine with newly synthesized class II MHC molecules (Davidson *et al.*, 1991). These peptide/MHC complexes then are transported to the cell surface, where they serve as recognition units for antigen-specific helper T cells.

Although it is attractive to think that AgR signaling reactions promote antigen uptake, the current evidence argues against this hypothesis. Presentation of tetanus toxoid to helper T cell lines by antigen-specific EBV-transformed B cell lines is 10^3–10^4 times more efficient than presentation by the corresponding nonspecific EBV-transformed B cell lines (Lanzavecchia, 1985). Similarly, presentation of rabbit immunoglobulins to specific T cells by B cells is enhanced 10^3- to 10^4-fold by using rabbit immunoglobulins that can bind to the AgR (rabbit anti-Ig) (Tony and Parker, 1985). Monovalent Fab antibody fragments present antigen as well as do bivalent $F(ab')_2$ fragments (Tony *et al.*, 1985). The latter are capable of triggering signaling reactions and capping, whereas the former are not. Thus, the constitutive recycling of AgRs seems to be the primary route of antigen uptake. Conversely, introduction of certain mutations into the transmembrane region of the μ heavy chain greatly decreases antigen presentation, without having an obvious effect on AgR signaling via calcium elevation (Shaw *et al.*, 1990).

Although AgR signaling does not promote antigen uptake, it does enhance the ability of the B cell to present antigen (Casten *et al.*, 1985). For example, anti-Ig induces increased expression of class II MHC

molecules (Mond *et al.*, 1981), and these newly synthesized MHC molecules appear to be primarily responsible for binding peptides in endosomes (Davidson *et al.*, 1991). Anti-Ig stimulation of B cells also increases their adhesiveness for other cells, probably via the LFA-1/ICAM-1 cell–cell adhesion molecules (Dang and Rock, 1991). Finally, anti-Ig also induces expression of the B7 molecule, which provides a costimulatory signal to helper T cells needed to promote a vigorous response (Freedman *et al.*, 1987; Freeman *et al.*, 1989). In the absence of B7 on the antigen-presenting cell, the helper T cell can go into an anergic state, probably reflecting tolerance (Liu and Linsley, 1992). Thus, AgR signaling increases the effectiveness of the B cell as an antigen-presenting cell for helper T cells in several ways.

B. Changes in Ion Movements after Stimulation of the AgR

As described earlier, PIP_2 breakdown triggered by ligation of the AgR induces release of Ca^{2+} from internal stores (probably the ER) into the cytoplasm. There is also an increase in Ca^{2+} flow into the cell through the plasma membrane (MacDougall *et al.*, 1988b). After the first few minutes of stimulation, this influx of Ca^{2+} from outside is required to sustain elevated intracellular calcium (Ransom *et al.*, 1986; LaBaer *et al.*, 1986; Gelfand *et al.*, 1989). The mechanism by which these Ca^{2+} channels are regulated in B cells is unknown, although there is evidence in T cells that they are also activated by IP_3 (Kuno and Gardner, 1987; Khan *et al.*, 1992). These channels appear to be inactivated by high intracellular Ca^{2+} levels (Gelfand *et al.*, 1989), a feature that would prevent intracellular free Ca^{2+} from rising too high.

Calcium influx is opposed by Ca^{2+} pumps that transport Ca^{2+} either into the intracellular storage site or across the plasma membrane. These Ca^{2+} pumps are probably responsible for the observed reduction of the initial peak in intracellular free Ca^{2+} to a plateau level, which is still elevated compared to the resting Ca^{2+} level. This plateau Ca^{2+} level is sustained while AgR-induced PIP_2 breakdown continues for at least an hour (Bijsterbosch *et al.*, 1985). The reduction from the peak Ca^{2+} level to the lower sustained level has also been interpreted as being due to receptor desensitization (Cambier *et al.*, 1988), and such a phenomenon could play a role in this process.

Elevation of intracellular Ca^{2+} is accompanied by a rapid and small hyperpolarization of the plasma membrane (LaBaer *et al.*, 1986; MacDougall *et al.*, 1988a; Gelfand and Or, 1991), although this has not been uniformly observed (Grinstein and Dixon, 1989; Lewis and Cahalan, 1990). As Ca^{2+} influx would depolarize the membrane somewhat, the hyperpolarization must reflect electrogenic flow of other

ions, probably K^+ ions traveling outward. Recently, evidence has been presented for the activation of Ca^{2+}-dependent K^+ channels on anti-IgM treatment of B cells (MacDougall *et al.*, 1988a; Mahaut-Smith and Schlichter, 1989). Hyperpolarization is also seen when intracellular Ca^{2+} is elevated with a calcium ionophore, and it is delayed when the rise in intracellular Ca^{2+} is delayed by increasing the Ca^{2+} buffering capacity of the cells (MacDougall *et al.*, 1988a). These phenomena support the hypothesis that elevation of intracellular Ca^{2+} leads to activation of a K^+ channel.

Other ionic movements are also triggered by anti-Ig stimulation of B cells. For example, there is evidence for an influx of Na^+ ions, based on increases in Na^+/K^+ pump activity (Heikkila *et al.*, 1983). This transporter pumps Na^+ back out to reestablish the proper ionic gradients that maintain the membrane potential, and its activity is thought to reflect a response to elevated intracellular Na^+ levels (see also Grinstein and Dixon, 1989).

It has also been reported that the plasma membrane becomes depolarized on treatment with anti-Ig (Monroe and Cambier, 1983a). This depolarization is much slower than the hyperpolarization mentioned above, as it takes 1–2 hours to reach maximum. In those experiments, membrane potential was measured by using cyanine dyes as probes of the potential. More recently, some problems with this technique have been noted, including concentration of these dyes in mitochondria (Grinstein and Dixon, 1989). Thus, the increased dye fluorescence may not solely reflect changes in membrane potential. However, it should be noted that artificial depolarization is able to mimic the induction of class II MHC molecules seen with anti-Ig stimulation, supporting the idea that there is a functionally significant prolonged depolarization in anti-Ig-stimulated B cells (Monroe and Cambier, 1983b). [For further discussion of this issue, see Grinstein and Dixon (1989).]

C. Induction of Gene Expression

Stimulation of the AgR of B cells leads to rapid induction of transcription of a series of genes. Among these are c-*myc*, c-*fos*, *egr-1* and *junB*, all of which are believed to be transcription factors (Kelly *et al.*, 1983; Monroe, 1988; Seyfert *et al.*, 1989; Tilzey *et al.*, 1991). The induction of these genes is not blocked by inhibitors of protein synthesis. Therefore, the machinery needed to initiate the transcription of these genes is preexisting in cells. For each of these genes, pharmacologic agents that mimic the phosphoinositide second messengers are capable of inducing their expression, suggesting that this is the signal transduction pathway responsible for their induction. In the case of

c-*myc* and *egr-1*, full induction can be achieved by treatment of cells with phorbol esters, indicating that protein kinase C is the primary route of induction (Klemsz *et al.*, 1989; Seyfert *et al.*, 1990). In the case of *egr-1*, the induction seems to be mediated by repeated serum response elements (SREs) upstream of the gene (Seyfert *et al.*, 1990). In contrast, full induction of c-*fos* in B cells requires elevation of intracellular calcium and activation of protein kinase C (Klemsz *et al.*, 1989; Mittelstadt and DeFranco, 1993).

Recently, seven additional genes that are induced by serum stimulation in quiescent fibroblasts were screened for induction by anti-Ig in B cells (Mittelstadt and DeFranco, 1993). Four of these genes (*nur77*, *nup475*, *pip92*, and *3CH134*) are induced by anti-Ig in B cells. Induction of expression of these genes is not blocked by protein synthesis inhibitors. Thus, they also qualify as early response genes. As with c-*myc* and *egr-1*, all four of these genes are induced by addition of phorbol esters, although in the case of *3CH134*, calcium elevation also plays a role under some circumstances. Three of these genes are also induced in the 2PK3 cell line, and here a transfected M1 muscarinic acetylcholine receptor, which stimulates phosphoinositide breakdown but not tyrosine phosphorylation (except for MAP kinase), is also able to induce appearance of these mRNAs (Mittelstadt and DeFranco, 1993). These observations indicate that physiological levels of phosphoinositide second messengers are capable of activating expression of these genes.

Many of the genes that are initially induced in response to growth factor stimulation of fibroblasts are thought to be transcription factors, which then are responsible for a second, more extensive wave of gene inductions. This concept is likely to have considerable validity for the B cell stimulated through the AgR as well, especially as most of the early response genes identified to date are known or putative transcription factors. Of these, the best understood is the c-*fos* gene product. Fos can form heterodimers with Jun and the Jun-related proteins via the leucine-zipper motif. Fos/Jun heterodimers bind to DNA sites called TPA-response elements (TREs) and are generally quite active at inducing transcription (Ransone and Verma, 1990). Jun is present prior to stimulation in most cells and, unlike Fos, can form homodimers. On protein kinase C stimulation, Jun homodimers can be active transcriptionally, although generally to a lesser extent than Fos/Jun heterodimers (Ransone and Verma, 1990; Boyle *et al.*, 1991). In B cells, anti-Ig stimulation does not lead to immediate appearance of protein complexes that can bind to TREs *in vitro* (Chiles *et al.*, 1991; Chiles and Rothstein, 1992). This observation suggests that TREs are not

involved in the induction of the immediate response genes and may be important for subsequent transcriptional regulatory events. After about 2 hours, anti-Ig-stimulated B cells do exhibit TRE-binding proteins. As c-*fos* mRNA peaks by 30 minutes after stimulation, it seems likely that these complexes represent heterodimers of Fos with Jun or one of the Jun-related proteins. Also induced by anti-Ig stimulation is Jun-B (Tilzey *et al.*, 1991). Jun-B acts as a inhibitor of Fos/Jun-activated transcription, at least under some circumstances (Chiu *et al.*, 1989). Whether Jun-B might act positively to stimulate transcription from some enhancers is unknown.

Anti-Ig stimulation of B cells also activates the NF-κB transcription factor. In other cell types, NF-κB is largely present in the cytoplasm as an inactive complex with the inhibitor I-κB. Stimulation of the cells leads to inactivation and dissociation of I-κB, after which the NF-κB goes to the nucleus and activates transcription (Lenardo and Baltimore, 1989). In B cells, some NF-κB is constitutively active and is believed to be important for expression of the Ig κ gene. B cells do have additional inactive NF-κB, however, and anti-Ig treatment leads to its activation (J. Liu *et al.*, 1991; Rooney *et al.*, 1991). The mechanism by which NF-κB is activated is not well established, although phorbol esters can lead to its activation in pre-B cell lines and PKC can phosphorylate and inactivate I-κB *in vitro* (Ghosh and Baltimore, 1990). In any case, NF-κB is a transcription factor that may mediate induction of the early response genes in response to anti-Ig. In support of that idea, c-*myc* induction in the WEHI-231 B cell line depends on an upstream DNA site that is an NF-κB binding site (Duyao *et al.*, 1990).

Another candidate for a transcription factor involved in early response gene induction in B cells is serum response factor (SRF) and the associated protein called ternary complex factor, or $p62^{TCF}$ (Berk, 1989). The *egr-1* gene has several SRF-binding sites (also called SRE, for serum response element) that are required for induction of this gene in response to anti-Ig stimulation (Seyfert *et al.*, 1990). Interestingly, MAP kinase can phosphorylate $p62^{TCF}$ *in vitro*, suggesting a mechanism for activation of transcription through SRE sites (Gille *et al.*, 1992).

Transcriptional activation events induced by NF-κB and SRF/$p62^{TCF}$ are thought to be downstream of protein kinase C. Transcriptional regulation of some genes induced by anti-Ig, such as c-*fos*, *3CH134*, and class II MHC genes, appears to involve Ca^{2+} elevation as well PKC activation (Monroe and Kass, 1985; Dennis *et al.*, 1987; Klemsz *et al.*, 1989; Mittelstadt and DeFranco, 1993). One transcription factor that could play a role in gene inductions involving Ca^{2+}

elevation is Ets-1. This transcription factor rapidly becomes phosphorylated on treatment of B cells with anti-Ig or Ca^{2+} ionophore (Fisher *et al.*, 1991). In mitogen-stimulated thymocytes, Ca^{2+} elevation leads to phosphorylation of Ets-1, whereupon it loses its DNA-binding ability (Pognonec *et al.*, 1989). If the phosphorylation of Ets-1 also causes loss of DNA-binding ability in B cells, then this would represent a Ca^{2+}-dependent change in the ability of Ets-1 to regulate transcription.

In summary, there are a number of early response genes that are induced by AgR signaling events acting on preexisting transcription factors. Most or all of these events appear to involve phosphorylation of transcription factors by serine/threonine protein kinases that are activated by a cascade of events (Fig. 3) (Hunter and Karin, 1992; Meek and Street, 1992). For example, AgR-triggered tyrosine phosphorylation induces activation of phospholipase C $\gamma 1$ and $\gamma 2$. These enzymes generate the second messengers IP_3 and diacylglycerol. The former messenger leads to elevation of intracellular free Ca^{2+}, which can activate calmodulin-dependent protein kinase II, which may act on Ets-1. The other phosphoinositide-derived second messenger, diacylglycerol, activates protein kinase C isozymes, which can activate MAP kinase, which in turn may regulate $p62^{TCF}$. In these ways, the early response genes may be induced. Some of these products of the early response genes are transcriptional regulators, and they then induce the expression of a second wave of genes.

VI. Mechanism of Contact-Dependent T Cell Help

Antibody responses elicited by soluble protein antigens are T cell dependent, and this dependence cannot be alleviated by addition of cell-free supernatants of activated T cells (Noelle and Snow, 1990). Responses to this class of antigens only occur if B cells are allowed to make contact with the T cells providing help. Presumably, soluble protein antigens are less proficient at inducing AgR signaling than are cell-bound or polysaccharide antigens and therefore an additional signal from helper T cells is required to support these responses. Moreover, this signal is apparently conveyed by a cell-bound molecule.

With the advent of cloned helper T cell populations, several investigators were able to define circumstances wherein helper T cells stimulate B cells polyclonally (DeFranco, 1987; Noelle and Snow, 1990). Activation of the T cells through the TCR confers on them the ability to activate resting B cells, regardless of the antigen specificity of the B cell. For example, culturing cloned helper T cells with a mixture of

antigen-presenting B cells and B cells that cannot present antigen led to similar activation of both B cell populations (DeFranco *et al.*, 1984). This conclusion was reinforced in a number of ways. For example, it is possible to activate T cells with anti-TCR antibodies, add them to resting B cells, and then observe strong stimulation of proliferation and antibody production by the B cells (Tite *et al.*, 1984; Julius *et al.*, 1988). In general, treatment of the B cells with anti-Ig enhances their responsiveness to activated T cells, but is not necessary to observe a vigorous response (Julius *et al.*, 1982; DeFranco *et al*, 1984).

As mentioned earlier, activation of B cells by antigen-activated helper T cells requires cell–cell contact. Moreover, the helper T cells can be lightly fixed with paraformaldehyde and still be effective (Noelle *et al.*, 1989). This observation suggested that activation of the T cell leads to expression of one or more T cell surface molecules that are responsible for stimulating the B cells. Indeed, plasma membranes isolated from activated, but not resting, T cells are also able to induce early events in B cell activation (Brian, 1988; Hodgkin *et al.*, 1990; Noelle and Snow, 1991). Strong proliferation and differentiation of B cells is observed in response to membranes from activated helper T cells if IL-4 and IL-5 are also included (Hodgkin *et al.*, 1990; Noelle *et al.*, 1991).

Recently, the molecular nature of this contact-dependent B cell activation signal has become apparent from convergence of these studies with studies on CD40, a protein first described as being present on the surface of human B cells (Clark, 1990). Human B cells exhibit a number of interesting responses following stimulation with anti-CD40 antibodies, including increased homotypic adhesion, increased survival, and long-term proliferation (Clark, 1990; Banchereau *et al.*, 1991). These observations led investigators to search for the ligand of CD40. Chimeric proteins were constructed that contained the extracellular domain of CD40 and the constant regions of IgG_1. These chimeras were found to bind to activated, but not resting, T cells and to block T cell-dependent activation (Armitage *et al.*, 1992; Noelle *et al.*, 1992; Fanslow *et al.*, 1992; Lane *et al.*, 1992). The molecule recognized by the CD40 chimera (called CD40L, for ligand of CD40) was cloned and found to be a 33- to 39-kDa type II membrane glycoprotein with homology to TNFα (Armitage *et al.*, 1992; Hollenbaugh *et al.*, 1992). Paraformaldehyde-fixed CV-1/EBNA cells expressing CD40L are able to stimulate proliferation of B cells, and these cells can be driven to secrete IgE in the presence of IL-4 (Armitage *et al.*, 1992; Hollenbaugh *et al.*, 1992; Spriggs, *et al.*, 1992). These observations suggest that CD40L is the molecule present in the membranes of

activated helper T cells responsible for activating B cells. Moreover, a mAb that binds to murine CD40L was isolated by immunizing with fixed, activated helper T cells and screening for ability to block T cell-dependent activation of B cells (Noelle *et al.*, 1992). Recently, it has been found that the gene encoding CD40L maps to the X chromosome, and that it is defective in patients with X-linked hyper-IgM syndrome (Aruffo *et al.*, 1993; Allen *et al.*, 1993; Korthauer *et al.*, 1993; DiSanto *et al.*, 1993; Fuleihan *et al.*, 1993). This immunodeficiency disease is characterized by normal or elevated IgM and little or no IgG or IgA. As production of IgG and IgA is characteristic of T cell-dependent antibody responses, the immunoglobulin levels in this disease likely reflect a severe defect in such responses. Thus, it is clear from a variety of lines of evidence that CD40L represents an important molecule involved in the stimulation of B cells by activated T cells.

The mechanism by which CD40 informs the cell that it has contacted CD40L is not well understood. Stimulation of resting B cells with fixed, activated helper T cells leads to activation of NF-κB, and this activation seems to be due to CD40L (Lalmanach-Girard *et al.*, 1993). In addition, treatment of human tonsillar B cells with anti-CD40 induces strong protein tyrosine phosphorylation, phosphoinositide breakdown, and activation of several serine/threonine protein kinases (Uckun *et al.*, 1991). These events are similar to what is seen on stimulation of B cells with anti-Ig. As both anti-Ig and CD40L can induce early activation events in B cells it will be interesting to compare the specific signaling targets of tyrosine phosphorylation induced in these two ways.

CD40L may not be the only T cell membrane protein that contributes to contact-dependent B cell activation. Although fixed CV-1/EBNA cells expressing CD40L can directly stimulate proliferation of resting B cells, CD40L expressed in COS cells and soluble CD40L only stimulate significant proliferation in the presence of phorbol myristate actetate (PMA) or anti-CD20 antibodies (Hollenbaugh *et al.*, 1992). Thus, activated T cells may also express other molecules that work in conjunction with CD40L to drive B cell proliferation. A wide variety of molecules exist on the surfaces of helper T cells and B cells that can interact with one another, and it is possible that some of these molecules also play important roles. In particular, CD72/Lyb2 on the B cell can interact with CD5 on the T cell (Van de Velde *et al.*, 1991), and this interaction could send important signals to the B cell since some anti-Lyb2 mAbs activate B cells to proliferate (Subbarao and Mosier, 1983) whereas other anti-Lyb2 mAbs block T cell help (Yakura *et al.*, 1981).

Another molecule that receives information during B cell/helper T cell interaction is the class II MHC molecule. Class II MHC molecules with the appropriate peptide bound are the ligand for the TCR and CD4. In addition, class II MHC molecules complexed with other peptides might bind to CD4 molecules while T cells and B cells are interacting. In efforts to simulate these interactions, B cells have been treated with anticlass II MHC antibodies. In resting B cells, these antibodies stimulate an increase in cAMP levels (Cambier *et al.*,1987). As helper T cell interaction with resting B cells also induces elevations in intracellular cAMP levels (Pollok *et al.*, 1991), signaling through class II MHC molecules may occur in helper T cell/B cell conjugates.

In contrast to the class II MHC signaling events seen in resting murine B cells, human B cells or murine B cells that have been previously stimulated with anti-Ig and IL-4 exhibit increases in protein tyrosine phosphorylation and PIP_2 breakdown in response to anticlass II MHC antibodies (Lane *et al.*, 1990; Cambier *et al.*, 1991). The pattern of protein tyrosine phosphorylation, as assessed by anti-P-tyr immunoblotting, is very similar to that seen following anti-Ig treatment. Apparently, previous encounter of the B cell with antigen changes the nature of signaling through the MHC class II molecule. The significance of this change is not entirely clear. One possibility is that this is a mechanism to modulate the response to CD40L depending on previous contact of the B cell with antigen. If a CD40L-bearing T cell interacts with a B cell that has not seen antigen, then the signal through the class II MHC molecule involves cAMP elevation, and this could inhibit B cell activation. On the other hand, if the B cell in question had already contacted its antigen, then interaction with the activated helper T cell would provide two stimulatory signals, the first via CD40L, the second via CD4 binding to class II MHC molecules, which now signal in a different mode.

An alternative possibility is that the rise in cAMP is needed for the resting antigen-presenting B cell to activate the helper T cell. This hypothesis comes from studies of antigen presentation by transfected B cell lines. Transfection of the M12 B lymphoma cell line with genes encoding α and β chains of class II MHC molecules allows these cells to present antigen to helper T cell via the introduced MHC molecules. Expression of class II MHC molecules with truncated cytoplasmic domains, however, allows antigen presentation to only some antigen-specific T cell hybridomas (Nabavi *et al.*, 1989). Similarly truncated class II MHC molecules fail to induce cAMP upon anticlass II antibody treatment (Wade *et al.*, 1989). These observations suggest that the defect in antigen presentation via the truncated class II MHC

molecules might be due to their inability to generate a cAMP signal on B cell/T cell contact. This hypothesis was supported by the observation that preincubation of the transfected M12 cells in dibutyryl cAMP restored the antigen-presenting capability of these cells (St-Pierre *et al.*, 1989).

Given the role of B7 as a costimulatory molecule for activation of helper T cells, B7 expression was examined (Nabavi *et al.*, 1992; T. H. Watts, *et al.*, 1993b). Incubation of M12 cells expressing wild-type class II MHC molecules with peptide and antigen-specific T cells leads to induction of B7 on the M12 cells. In contrast, M12 cells expressing truncated class II MHC molecules fail to show this response. Moreover, cAMP induces B7 expression on the B cell surface, and transfection of the M12 cells with a B7 expression vector also rescued the antigen-presenting ability of the truncated class II MHC molecules (Nabavi *et al.*, 1992; T. H. Watts *et al*, 1993). The most straightforward explanation of these findings is that T cell recognition of appropriate antigenic-peptide/MHC class II molecules on the B cell causes the latter molecules to send a cAMP signal which induces B7 expression. Once induced, the B7 can act as a costimulatory molecule by interacting with CD28 or CTLA-4 on the T cell surface, leading to strong T cell activation. These studies have been carried out in a B lymphoma cell line, so their relevance to normal B cells is not clear. Recently, however, it has been found that anticlass II MHC antibodies do induce B7 expression in normal human B cells (Koulova *et al.*, 1991), suggesting that this series of events is also applicable to normal B cells.

VII. Signaling by Receptors for B Cell Growth and Differentiation Factors

As discussed earlier, there appears to be two primary ways in which resting B cells become stimulated to undergo early activation events: strong stimulation through the AgR by a multivalent or cell-bound antigen, and stimulation through CD40 by contact with an antigen-activated helper T cell. Once the B cell has committed itself to early activation, it also becomes ready to proliferate and differentiate in response to a variety of cytokines, including IL-2, IL-4, IL-5, IL-6, and IFN-γ (Kishimoto and Hirano, 1988; Vitetta *et al.*, 1989). Recent work has provided great insight into the structures of these cytokines and into the nature of their receptors. Information regarding the signaling properties of these receptors is also starting to become available, particularly for the IL-2 receptor (Taniguchi and Minami, 1993).

A. Cytokine Receptor Structure

Most of the receptors for cytokines regulating B cell activation exhibit homology in their extracellular domains, and thus are members of a group called the cytokine receptor or hematopoietin receptor family (Miyajima *et al.*, 1992; Bouley and Paul, 1992). The basic homology unit of this family consists of two adjacent 100-amino acid domains, each of which is postulated to form a β-pleated sheet structure similar to an immunoglobulin domain (Bazin, 1990). This prediction has been roughly born out for the growth hormone receptor, the one member of this family for which a structure has been determined (de Vos *et al.*, 1992). This structure is composed of seven antiparallel β-strands forming two side-by-side sheets. One loop between neighboring β-strands contains a highly conserved WSxWS motif (where W is tryptophan, S is serine, and x is any amino acid). Within this framework of an evolutionarily conserved unit, there is surprising variability in receptor structure. For example, the IL-2R has three polypeptides (α, β, and γ), two of which are members of this family (Minami *et al.*, 1993). In contrast, the IL-5R and IL-6R are two chain structures, with both chains being members of the family (Miyajima *et al.*, 1992). For these latter two receptors, the α chain is primarily responsible for ligand binding. Expression of the α chains in cells lacking β chains typically yields only low-affinity binding. These observations indicate an effect of the β chain on either binding or α chain structure. Interestingly, the β chains of these receptors are shared with other cytokine receptors (IL-3R and GM-CSFR in the case of the IL-5R β chain, and the leukemia inhibitory factor receptor, the oncostatin M receptor, and the receptor for ciliary neurotrophic factor in the case of the IL-6R β chain) (Bouley and Paul, 1992). In the case of the IL-6R, it is clear that the β chain is responsible for signal transduction, as the α chain can be successfully supplied in the form of a soluble extracellular domain (Taga *et al.*, 1989). Finally, the IL-4R is a member of this family but appears to function as a single chain (Fig. 5).

Considerably less is known about the signaling properties of the cytokine receptors, although recent evidence suggests that they may all signal by activating intracellular protein tyrosine kinases (Miyajima *et al.*, 1992; Taniguchi and Minami, 1993), as discussed next in the context of individual cytokine receptors. In particular, the signaling properties of the IL-2R, the IL-4R, and the IL-6R will be described.

B. IL-2 Receptor Signaling

Among the actions of IL-2 in B cells is promotion of differentiation to the antibody-secreting cell stage (Nakanishi *et al.*, 1983, 1984). This

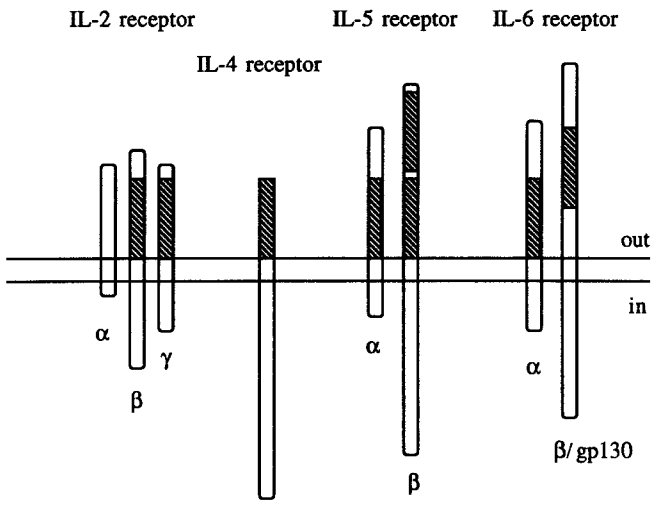

FIG. 5. Receptors for B cell growth and differentiation factors. The structures of some of the receptors for cytokines that stimulate B cell growth or differentiation are shown. The cytokine receptor homology domain of approximately 200 amino acids (see text) is indicated by the striped regions.

includes a number of regulatory changes. One that has been studied in detail is the induction of the gene for J chain, which joins IgM into pentamers. IL-2 induces J chain gene transcription (Blackman *et al.*, 1986) and this regulation occurs via both positive-acting transcription factors that are stimulated by IL-2R signaling and negative-acting repressive elements that are inactivated (Lansford *et al.*, 1992). IL-2 can also stimulate the proliferation of B cells that have been activated, for example, with anti-Ig (O'Garra *et al.*, 1988).

The receptor for IL-2 is particularly well studied and a great deal is known about its signaling properties. It should be noted that most experiments on IL-2 signaling have been done in T cells or in transfected nonlymphoid cells, so whether events seen in those contexts also occur in B cells often remain to be determined. The IL-2R does not induce elevation of cAMP or breakdown of PIP_2 (Mills *et al.*, 1986; Tigges *et al.*, 1989). Moreover, protein kinase C is not required for the action of this receptor (Mills *et al.*, 1988; Valge *et al.*, 1988). Stimulation of IL-2 receptor-expressing cells with IL-2 does induce the tyrosine phosphorylation of a number of polypeptides (Saltzman *et al.*, 1988), including the β chain of the receptor (Mills *et al.*, 1990). IL-2 stimulation leads to activation of PI 3-kinase (Remillard *et al.*, 1991; Augustine *et al.*, 1991; Merida *et al.*, 1991) and tyrosine phosphoryla-

tion of the Raf-1 serine/threonine protein kinase (Turner *et al.*, 1991). Raf-1 is an upstream activator of MAP kinase in several cell types (Dent *et al.*, 1992; Kyriakis *et al.*, 1992), but IL-2 does not activate MAP kinase (Kuo *et al.*, 1992), whereas the AgRs of B cells and T cells do (Casillas *et al.*, 1991; Gold *et al.*, 1992a). Thus, the functional consequences of tyrosine phosphorylation of Raf-1 in response to IL-2 are presently unclear.

IL-2 addition to B cells or to T cells induces the hydrolysis of phosphatidylinositol-glycan compounds (Merida *et al.*, 1990; Eardley and Koshland, 1991), a reaction first observed in cells treated with insulin (Saltiel *et al.*, 1986). As the insulin receptor has a tyrosine kinase-containing cytoplasmic region, it is likely that this signaling reaction is triggered by a tyrosine phosphorylation event. Addition of the phosphoinositol-glycan products of this signaling reaction to an IL-2-dependent T cell line enhanced the responsiveness of these cells to IL-2 (Merida *et al.*, 1990). The most straightforward interpretation of this result is that these compounds are one of several important second messengers generated in response to IL-2 and, moreover, in these cells, they are limiting second messengers at low IL-2 concentrations.

Another signaling reaction induced by IL-2 is an increase in the fraction of GTP bound to $p21^{ras}$ (Satoh *et al.*, 1991; Graves *et al.*, 1992). The mechanism by which IL-2R induces this increase is not clear; *in vitro* experiments did not detect a change in overall Ras-GTPase activity following IL-2 treatment, whereas a decrease in GTPase activity was seen following treatment with anti-TCR antibodies or phorbol esters (Graves *et al.*, 1992). Note that tyrosine phosphorylation of *ras*GAP was not observed in response to IL-2. Although the mechanism by which IL-2 affects $p21^{ras}$ is unclear, this effect is also commonly caused by the growth factor receptors that have tyrosine kinase domains in their cytoplasmic regions (Cantley *et al.*, 1991), suggesting that IL-2R may also mediate this effect via protein tyrosine phosphorylation. Therefore, IL-2 induces a number of signaling events seen in response to ligand binding to other receptors, particularly those containing intrinsic tyrosine kinase activities. Thus, it seems likely that most or all of IL-2R signaling involves activation of one or more intracellular protein tyrosine kinases.

Candidates for the kinases activated by the IL-2R include $p56^{lck}$ and the newly described intracellular kinase Itk (Siliciano *et al.*, 1992). The latter protein kinase was discovered by cDNA cloning using homology to known protein kinases. Its expression in T cells is enhanced in response to IL-2, which suggests a role in IL-2R signaling as IL-2 also induces much greater expression of the receptor α subunit. Al-

though Itk is not expressed in B cells, B cells do express a very similar tyrosine kinase called Btk, the gene for which is defective in X-linked agammaglobulinemia (Vetrie *et al.*, 1993; Tsukada *et al.*, 1993).

A role for $p56^{lck}$ in signaling by the IL-2R is suggested by the observation that adding IL-2 to T cells stimulates $p56^{lck}$ kinase activity (Horak *et al.*, 1991), and that this kinase associates with the IL-2R β chain (Hatakeyama *et al.*, 1991). This interaction is mediated by the kinase domain of $p56^{lck}$ interacting with the acidic region of the IL-2R β chain. Curiously, this region of the IL-2R β chain is not essential to the stimulation of growth in transfected BAF-B03 pro-B cells, whereas another region of the β chain, the serine-rich region, is required for IL-2 to stimulate the growth of these IL-3-dependent cells (Hatakeyama *et al.*, 1989). Both of these two regions may cooperate in some fashion to activate $p56^{lck}$. Deletion of either region of the receptor β chain abolishes IL-2-induced tyrosine phosphorylation of the major tyrosine phosphorylation substrate, p85, and the increase in GTP bound to $p21^{ras}$ (Satoh *et al.*, 1992). A further complication is that $p53/56^{lyn}$ can also associate with the IL-2R and be activated by IL-2 (Torigoe *et al.*, 1992). $p53/56^{lyn}$ is more prevalent than $p56^{lck}$ in most B cells, although many B cells do express $p56^{lck}$ (Law *et al.*, 1992; Campbell and Sefton, 1992). The structural basis of the association of $p53/56^{lyn}$ with the IL-2R is not yet known. Nonetheless, the fact that the deletion of the acidic region does not abrogate the ability of the IL-2R β chain to stimulate growth of BAF-B03 cells suggests that the IL-2R can stimulate a second distinct signaling pathway and that this second pathway is sufficient to stimulate growth of these cells. This hypothesis is supported by examination of early response genes induced in response to IL-2 in BAF-B03 cells transfected with IL-2R β chain mutants (Shibuya *et al.*, 1992). The wild-type IL-2R stimulates transcription of c-*myc*, c-*fos*, and c-*jun*, and the mutant receptor missing the acidic region can still induce production of c-*myc*. Thus, induction of c-*fos* and c-*jun* and the increase in activation of $p21^{ras}$ appear to require the $p56^{lck}$–tyrosine phosphorylation pathway, whereas the induction of c-*myc* appears to require a second signaling pathway.

An interesting probe into the mechanism of IL-2R signaling is the immunosuppressant rapamycin. This agent inhibits the ability of T cells to proliferate in response to IL-2. It appears to do this by interacting with the FK506 binding protein FKBP12. As with FK506, rapamycin is thought to mediate its action by forming a complex with FKBP12. In any case, rapamycin appears to inhibit IL-2 action by blocking the activation of a serine/threonine protein kinase called

$p70^{S6K}$ (Kuo *et al.*, 1992). This is one of two protein kinases that can phosphorylate ribosomal protein S6, an event frequently correlated with stimulation of cell growth. It is unclear at this point whether rapamycin blocks the step in IL-2R signaling directly upstream of $p70^{S6K}$ or whether it acts further upstream. Moreover, these events have not yet been studied in B cells stimulated with IL-2.

C. IL-4 Receptor Signaling

Interleukin 4 enhances the survival of B cells *in vitro*, causes increased expression of class II MHC molecules, induces proliferation of B cells stimulated with anti-Ig, promotes antibody responses under a number of circumstances, and induces IgH class switching to IgG_1 and IgE (O'Garra *et al.*, 1988; Vitetta *et al.*, 1989). The mechanisms by which these varied biological effects are mediated are not understood. The interleukin 4 receptor is a single-chain member of the cytokine receptor family (Bouley and Paul, 1992). It has a large intracellular domain of about 500 amino acid residues, but lacks any discernible catalytic domain. Initial studies demonstrated that IL-4 does not induce PIP_2 breakdown or calcium elevation in murine B cells (Justement *et al.*, 1986; Mizuguchi *et al.*, 1986; O'Garra *et al.*, 1987). In contrast, IL-4 induces a short pulse of IP_3 production and a brief elevation of intracellular Ca^{2+} in human tonsillar B cells (Finney *et al.*, 1990). The elevation of Ca^{2+} is still observed in the absence of extracellular Ca^{2+} , indicating that it arises from intracellular stores. The short duration of this response may reflect an inability to mobilize Ca^{2+} entry through the plasma membrane. The short burst of PIP_2 breakdown and Ca^{2+} elevation is followed by an increase in cAMP levels. In contrast to the rapid response to receptors that utilize Gs to activate adenylyl cyclase (e.g., β-adrenergic receptor), this response takes about 10 minutes before it begins. Moreover, blocking the IL-4 induced Ca^{2+} elevation by buffering intracellular Ca^{2+} levels with BAPTA also blocks the cAMP response, indicating that the Ca^{2+} elevation is necessary for the increase in cAMP levels to occur. The increase in cAMP levels is not simply a consequence of the Ca^{2+} elevation, however, as Ca^{2+} ionophores do not trigger cAMP increases on their own. Interestingly, the production of soluble CD23 in response to IL-4 can be mimicked by a short pulse of phorbol dibutyrate and ionomycin, followed by dibutyryl cAMP (Finney *et al.*, 1990). Thus, these signaling events may have important downstream consequences in human B cells. Why these signaling events are seen in human B cells and not in murine B cells is not known.

In murine B cells and IL-4-responsive mast cell lines, IL-4 also

induces tyrosine phosphorylation of several proteins (Morla *et al.*, 1988; Wang *et al.*, 1992). Among the proteins that become tyrosine phosphorylated is the IL-4 receptor (Wang *et al.*, 1992). Tyrosine phosphorylation of a receptor is a common feature of receptors that trigger tyrosine phosphorylation-based signaling events. A major substrate of IL-4-induced tyrosine phosphorylation is a protein of 170 kDa called IL-4-induced phosphotyrosine substrate, or 4PS. The 4PS protein becomes tyrosine phosphorylated in response to IL-4 in mast cells, B cells, and T cells (Wang *et al.*, 1992; I. van Oostveen and M. R. Gold, unpublished observations). In mast cells, IL-4 stimulation leads to association of PI 3-kinase with 4PS (Wang *et al.*, 1992) and the appearance of the 3-phosphorylated phosphoinositide products of PI 3-kinase (Gold *et al.*, 1993d). In this regard, 4PS is reminiscent of IRS-1, which also becomes tyrosine phosphorylated and binds to PI 3-kinase after insulin stimulation (Backer *et al.*, 1992). 4PS is not IRS-1, but it may be a close homologue. It will be interesting to see if IL-4 also activates PI 3-kinase in B cells, as assessed by changes in the amounts of 3-phosphorylated inositol-containing phospholipids. If so, this would represent overlap between the signaling of the IL-4 receptor and of the B cell AgR. Other targets of AgR-induced tyrosine phosphorylation, such as PLC-γ and *ras*GAP (see above), are not tyrosine phosphorylated in response to IL-4, at least not in mast cells (Wang *et al.*, 1992).

D. IL-6 Receptor Signaling

Interleukin 6 is a terminal differentiation factor for B cells and a growth factor for murine plasmacytoma cells (Kishimoto *et al.*, 1992). The IL-6 receptor is composed of two polypeptides that are members of the cytokine receptor family (Kishimoto *et al.*, 1992). The 80-kDa chain of the receptor (IL-6Rα) is responsible for ligand binding. Removal of the transmembrane and cytoplasmic domains yields an extracellular soluble molecule that can still bind IL-6. In addition, the complex of IL-6 and soluble IL-6Rα can confer IL-6 responsiveness on cells that lack IL-6Rα expression. The presence of IL-6 induces IL-6Rα to form a complex with another polypeptide called gp130 (Taga *et al.*, 1989). These observations led to the hypothesis that gp130 is the signal-transducing component of the IL-6R. The gene encoding gp130 has been cloned and found to be a new member of the cytokine receptor family (Kishimoto *et al.*, 1992). In addition, gp130 (or IL-6Rβ chain) can also participate in responses involving other cytokine receptors. These observations have led to the view that IL-6 binding to the IL-6Rα chain induces it to associate with gp130, which then transduces signals into the interior of the cell.

Among the early events following IL-6 binding are phosphorylation of the gp130 chain (Kishimoto *et al.*, 1992) and an increase in the GTP-bound state of $p21^{ras}$ (Nakafuku *et al.*, 1992). Presumably, the IL-6R activates protein tyrosine phosphorylation-signaling reactions, as described previously for the B cell AgR, the IL-2R, and the IL-4R. In addition, there is activation of a basic leucine-zipper transcription factor called NF-IL-6, which has been implicated in the induction of transcription of acute-phase genes in hepatocytes, cytokine genes in macrophages, and possibly immunoglobulin genes in B cells (Akira and Kishimoto, 1992). Expression of an activated mutant form of $p21^{ras}$ in 3T3 fibroblasts and P19 embryonic carcinoma cells induces the activity of introduced NF-IL-6 (Nakajima *et al.*, 1993). Transcriptional activation is associated with phosphorylation of the protein on a threonine residue that is embedded in a MAP kinase consensus sequence (Nakajima *et al.*, 1993). MAP kinase will phosphorylate NF-IL-6 *in vitro* on this site. Moreover, mutation of this residue abrogates the ability of activated *ras* to stimulate transcription via NF-IL-6 (Nakajima *et al.*, 1993). These observations support a model whereby the IL-6R activates $p21^{ras}$, which leads to activation of MAP kinase, which in turn phosphorylates the NF-IL-6, inducing it to activate transcription. How much of this pathway will be applicable to the ability of IL-6 to induce terminal differentiation of activated B cells is not clear. IL-6 does induce transcriptional activation of two early response genes, *junB* and *TIS 11* (Nakajima and Wall, 1991), as well as immunoglobulin heavy chain genes in B cell lines (Raynal *et al.*, 1989).

VIII. B Cell Survival

It has become apparent that the regulation of proliferation is not the only way in which B cell populations are regulated. Rather the control of cell survival is also a key step in regulation of B cell antibody responses (Cohen *et al.*, 1992). In particular, the protooncogene *bcl-2* has received considerable attention as a molecule that plays an important role in mediating B cell survival (Korsmeyer, 1992; DeFranco, 1992a).

The *bcl-2* gene was initially isolated by virtue of translocations between the *bcl-2* locus and the Ig loci seen consistently in follicular B cell lymphomas (Korsmeyer, 1992). Several experiments suggest that *bcl-2* does not promote cell proliferation, but rather promotes cell survival. For example, introduction of *bcl-2* into an IL-3-dependent pre-B cell line does not abrogate IL-3 dependence for growth, but does prevent rapid cell death on factor withdrawal (Vaux *et al.*, 1988).

Germinal center B cells undergo rapid expansion and somatic mutation of their immunoglobulin genes. These mutations create a few cells with AgRs of higher affinity and many cells with AgRs of lower or destroyed affinity for antigen. And yet, the average affinity of an antibody response increases with time. How are the cells of higher affinity selected from the cells with diminished affinity? It now appears that this occurs not by preferential expansion of cells with favorable mutations, but rather by selective survival of these cells after the proliferative period. These postproliferative "centrocytes" are present in an area of the germinal center that has the antigen-bearing follicular dendritic cells. Culture of isolated germinal center B cells *in vitro* reveals a high rate of cell death (Liu *et al.*, 1989). The survival of these cells is greatly enhanced by treatment with anti-Ig, anti-CD40 or, even better, the combination of the two. Interestingly, only a small fraction of centrocytes expresses *bcl-2* and treatment of these cells *in vitro* with anti-Ig or anti-CD40 induces its expression (Y.-J. Liu *et al.*, 1991). A favored model is as follows. Germinal center B cells rapidly proliferate and mutate their Ig genes. Next, the germinal center B cells stop growing and undergo antigen-based selection for AgRs that have gained or at least not lost affinity. Antigen contact with AgRs can induce *bcl-2* via signaling reactions and in this way mediate cell survival. Alternatively, the AgR can take up the antigen, after which the B cell can present antigen to helper T cells and receive via CD40 a signal to induce *bcl-2* and survive.

Cell survival also seems to be an important step in the propagation of B cell memory. Memory B cells can survive for long periods of time in the absence of proliferation (Schittek and Rajewsky, 1990). This survival, however, probably requires contact with antigen, since adoptive transfer of purified memory B cells to unimmunized hosts leads to rapid loss of memory. This loss of memory is prevented if the memory B cells express *bcl-2* due to presence of an IgH enhancer-driven *bcl-2* transgene (Nunez *et al.*, 1991). Thus, memory B cells seem to behave similarly to the centrocytes of germinal centers: antigen contact is needed to prevent cell death, and *bcl-2* expression is sufficient for survival.

Interestingly, cell death also seems to play an important role in the termination of an antibody response. Immunization of transgenic mice that constitutively express *bcl-2* under the control of the IgH enhancer leads to greatly prolonged antibody responses, elevated serum immunoglobulin levels, and the development of autoimmunity (Strasser *et al.*, 1991). From these experiments, it seems likely that antibody-secreting cells normally terminate antibody secretion by undergoing

programmed cell death, and if this is blocked by *bcl-2* expression, then these cells continue to secrete antibodies for a prolonged period of time.

IX. Summary

The activation of B lymphocytes from resting cells proceeds from the events of early activation to clonal proliferation to final differentiation into either an antibody-secreting plasma cell or a memory B cell. This is a complex activation process marked by several alternative pathways, depending on the nature of the initial antigenic stimulus. Over the past 5–10 years, there has been an explosion of studies examining the biochemical nature of various steps in these pathways. Some of that progress is reviewed here. In particular, we have described in detail what is known about the structure and function of the AgR, as this molecule plays a pivotal role in B cell responses of various types. We have also reviewed recent progress in understanding the mechanism of action of contact-dependent T cell help and of the cytokine receptors, particularly the receptors for IL-2, IL-4, and IL-6. Clearly, all of these areas represent active areas of investigation and great progress can be anticipated in the next few years.

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CD5 B Cells, a Fetal B Cell Lineage

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I. Introduction

Appreciation of heterogeneity in B cells has progressed substantially in the past decade based on investigation of the expression of a diverse array of surface markers. Earlier morphological and anatomical analyses had clearly discriminated B cells in the primary follicles of spleen from those in the marginal zone and germinal centers so that one could then ask whether expression of distinctive combinations of surface markers might provide an alternative way to delineate these populations. Studies showed that primary follicles largely consist of $IgM^{+}IgD^{++}$ cells, whereas marginal zone cells are $IgM^{++}IgD^{-}$ (Kroese *et al.*, 1990; Gray *et al.*, 1982). Germinal center B cells have been discriminated based on their high-level expression of the lectin, peanut agglutinin, along with unique levels of expression of several other cell surface molecules (Butcher *et al.*, 1982). However, the cell dynamics and precursor/progeny relationships among many of these phenotypically distinct subsets remain only incompletely understood. Although this heterogeneity has been interpreted as representing differences in stages of cell maturation or activation state in most cases, it now appears that some may represent distinctive subsets of mature B cell populations with different developmental origins.

In this review we cover progress in our understanding of an infrequent B cell subset, $CD5^{+}$ B cells (referred to hereafter simply as "CD5 B cells"), initially identified 10 years ago as "Ly-1 B" in mice (Hayakawa *et al.*, 1983). After a short background of CD5 expression on B cells, we focus on the issue of the relationship of CD5 B cells to B cell development, proposing a model that views this subset as the progeny of a fetal B cell differentiation pathway. Then we review several features of this population that distinguish it from other B cells, including a possible relationship between the normal population and the numerous $CD5^{+}$ B cell neoplasias. Next we discuss another feature that has evoked much interest, an apparent bias toward expressing autoreactive specificities along with skewed V gene usage. Finally, we briefly touch on work with the putative homologous human B cell subset, highlighting similarities and current work.

II. Background

A. Initial Reports of Expression of the Pan-T Cell CD5 Marker on B Cells

The expression of a T cell differentiation antigen on certain B cell tumors was first reported in a human leukemia study in 1978 (Boumsell *et al.*, 1978). This expression was found uniquely limited to Ig^+ cells in B chronic lymphocytic leukemia (B CLL) (and B lymphocytic lymphoma) and was not detected on Ig^+ cells in acute B leukemia, Burkitt's lymphomas, or other B cell proliferative disorders (Boumsell *et al.*, 1980; Wang *et al.*, 1980; Royston *et al.*, 1980; Kamoun *et al.*, 1981). Later, the antigen was determined to be CD5 (Wang *et al.*, 1980). In the mouse, Lanier *et al.* (1978, 1981) found that CD5 was expressed on several B cell lines (in the CH series) from B10.H-2^aH-4^b mice. The level varied among CH lines; however, subsequent more sensitive analysis strongly suggested that almost all of the CH lines expressed CD5 (Haughton *et al.*, 1986). In support of this high incidence of CD5 expression on late-appearing B lymphomas, Davidson *et al.* (1984) found that almost all cell lines derived from individual B lineage lymphomas occurring in NFS/N v-congenic mice express CD5.

Following the detection of CD5 on malignant B cells, initial attempts to detect B cells with this phenotype in normal adults were equivocal, because of the apparent paucity of such cells (Boumsell *et al.*, 1978, 1980; Wang *et al.*, 1980). However, utilizing a characteristic shared by B CLL and a subset of normal B lymphocytes, of forming rosettes with mouse erythrocytes, Caligaris-Cappio *et al.* (1982) reported the infrequent presence of CD5-positive B cells in human peripheral lymphoid tissues. The presence of similar cells in mice was also suggested by Ledbetter *et al.* (1980). Finally, improvements in multiparameter analysis using the fluorescence-activated cell sorter conclusively demonstrated the presence of these cells as normal constituents of the both murine and human lymphoid compartments (Hardy *et al.*, 1982; Manohar *et al.*, 1982; Hayakawa *et al.*, 1983; Antin *et al.*, 1986; Hardy and Hayakawa, 1986). The CD5 homologue in rabbit has been identified and shown to be expressed on most B cells in this species (Raman and Knight, 1992).

B. The CD5 Molecule

Homology between human and mouse CD5 molecules had been predicted from their distribution on several cell types and from their similar molecular mass (67 kDa) (Wang *et al.*, 1980; Lanier *et al.*, 1981; Ledbetter *et al.*, 1981). cDNA clones have been isolated for both

human (Jones *et al.*, 1986) and mouse (Huang *et al.*, 1987) CD5 and their sequences show 63% similarity overall, with strong (90%) homology in their carboxy-terminal regions and conservation of a cystein-rich amino-terminal region (Huang *et al.*, 1987; Jones *et al.*, 1986). Although a protein homology search failed to detect strong similarity with any other sequence in the available databases, CD5 is considered a distant member of the immunoglobulin gene superfamily because of predicted secondary structure similarities in the first amino-terminal subregion and identities between certain conserved residues (Huang *et al.*, 1987). Furthermore, both human and mouse CD5 show similar binding specificity: CD5 was found to bind to CD72 in humans, and its homologue, Lyb-2, in mice (Van de Velde *et al.*, 1991; Luo *et al.*, 1992).

Expression of CD5 on B cell lines has been confirmed both by immunoprecipitation (Martin *et al.*, 1981; Lanier *et al.*, 1981) and by Northern blot analysis (Huang *et al.*, 1987). That the same CD5 gene is expressed on normal T and CD5 B cells in mice has been further confirmed by the homologous gene targeting method (Tarakhovsky *et al.*, 1993) and by RT-PCR analysis (R. Hardy and K. Hayakawa, 1993, unpublished). Because Lyb-2/CD72 is present on all B lineage cells from the early precursor stage, and antibodies to this molecule can stimulate B cells (Subbarao and Mosier, 1983), it is interesting to speculate that the CD5/CD72 interaction may mediate T cell/B cell collaboration. However, the functional implication of CD5 expression on B cells remains an open question.

III. Origins of CD5 B Cells

A. Early Ontogenic Appearance and Peritoneal Cavity Localization

CD5-expressing B cells in mice have been detected in spleen cells at a very low frequency (1–2%) in most adult inbred strains (Hayakawa *et al.*, 1983). They are very infrequent in the spleen and peripheral blood, but curiously enriched among the B cells found in the peritoneal cavity, where they range from 10 to 40% of total cells (Hayakawa *et al.*, 1989a). In contrast, CD5 B cells are normally undetectable in bone marrow, lymph nodes, Peyer's patches, and the thymus (Hayakawa *et al.*, 1983, 1986a,b). There has also been a report that the very infrequent B cell population present in the thymus is $CD5^+$ (Miyama *et al.*, 1988), although this observation awaits further confirmation because another group failed to find this (Farinas *et al.*, 1990). During ontogeny, CD5 B cells constitute a relatively high proportion (30%) of splenic and peritoneal cavity IgM^+ B cells during the neonatal stage (1

week) (Hayakawa *et al.*, 1983; Haughton *et al.*, 1986), with peritoneal cavity retaining this high percentage into adulthood. These CD5 B cells in mice are consistently characterized by low levels of CD5 and low (or undetectable) levels of IgD ($IgD^{\pm}$). In comparison, $CD5^-$ B cells are found in typical lymphoid organs in increasing numbers with development, the majority of which eventually express high levels of IgD (IgD^{++}).

An enrichment of CD5 B cells in unique anatomical locations has also been reported in humans: CD5 B cells have been detected in the human peritoneal cavity in the early fetus (Bofill *et al.*, 1985). The mechanism that maintains high levels of CD5 B cells in the murine peritoneal cavity remains unclear and several hypotheses have been proposed. It should be noted that CD5 B cells are not simply "peritoneal cavity homing B cells" since variable numbers of IgD^{++} B cells are present in this location and, conversely, the number of CD5 B cells in spleen is comparable to that recovered from the peritoneal cavity.

B. Generation of CD5 B Cells from Fetal B Progenitors

Bone marrow is considered to be the primary site for B cell development in adult mice. Surprisingly, cell transfer experiments showed that CD5 B cells, in particular, are poorly generated from adult bone marrow as compared to fetal or newborn liver or bone marrow from young mice (Table I) (Hayakawa *et al.*, 1985). This paucity of CD5 B cell generation from adult bone marrow has been confirmed in analyses of >100 recipients (Lalor *et al.*, 1989b). The occasional studies published to date purporting to demonstrate CD5 B cell repopulation in adult recipients from adult bone marrow either consider $CD5^-$ B cells in the peritoneal cavity as equivalent to $CD5^+$ cells (Lalor *et al.*, 1989b) or else appear to suffer from a lack of good flow cytometry sensitivity, lack of appropriate staining controls, or both.

This restriction of CD5 B cell generation to early B cell precursor sources is not due to the presence of cells that influence the induction of CD5 on B cells. As Kantor *et al.* (1992) demonstrated, when animals were reconstituted with mixtures of fetal liver and bone marrow, wherein an allotype difference allowed discrimination of their progeny, then CD5 B cells were restricted to the fetal liver precursors. Furthermore, reconstitution of recipient mice using fractions sorted to enrich for hematopoietic stems cells (HSCs) using the criteria of Thy-1^{lo}/lineage$^-$ (Muller-Sieburg *et al.*, 1986; Spangrude *et al.*, 1988), corresponding to 0.2% of adult bone marrow and fetal or newborn liver, also revealed that numerous CD5 B cells are produced if liver is used, but few if bone marrow is the source (Hardy and Hayakawa,

TABLE I

DEVELOPMENTALLY REGULATED POTENTIAL FOR GENERATION OF PHENOTYPICALLY DISTINCT B CELL SUBSETS[a]

Type of Experiment	Cell Source	Ontogenic Timing	Cell Fraction	B Cell Phenotype Generated	
				$IgD^{\pm}CD5^{+}$	$IgD^{++}CD5^{-}$
Cell transfer	Liver	gs. d 13–14	Total	+	+
	Liver	Newborn	Total	+	+
	Bone marrow	1 month	Total	±	+
	Bone marrow	3 months	Total	−	+
	Liver	gs. d 15–16	Stem cell	+	+
	Liver	Newborn	Stem cell	+	+
	Bone marrow	3 months	Stem cell	−	+
	Liver	gs. d 15–16	Pro-B	+	−
	Liver	Newborn	Pro-B	+	+
	Bone marrow	3 months	Pro-B	−	+
	Omentum	gs. d 13	Total	+	−
Cell culture	Liver	gs. d 15–16	Pro-B	+	n.a.
	Bone marrow	3 months	Pro-B	−	n.a.
	Liver	Newborn	$L^{-}T^{+}aGM_1^{+}$	+	n.a.

[a] Key: gs. d, gestational day; stem cell, $Thy\text{-}1^{lo}Lin^{-}$; pro-B, progenitor B cell fraction, $B220^{+}CD43^{+}HSA^{+}$; $L^{-}T^{+}aGM_1^{+}$, $Lin^{-}Thy\text{-}1^{+}asialoGM_1^{+}$ fraction; n.a., not appropriate (IgD^{++} cells are rarely generated *in vitro*).

1992). These data imply that the difference in B cell development is intrinsic to the hematopoietic stem or early progenitor cells.

However, repopulation using HSCs cannot resolve putative restricted progenitors for CD5 B cells in the fetus, because the stem cell is capable of extensive self-renewal capacity and so could potentially colonize the bone marrow of recipients, giving rise to a fully reconstituted animal similar to normal ontogenic development. The generation of both $CD5^+$ and $CD5^-IgD^{++}$ B cells from fetal liver can be explained by this rationale. To address this possibility, an alternative approach is to use B cell lineage-committed progenitors that do not have significant self-renewing potential. In this case, recipient mice should possess the ability to permit long-term survey without full reconstitution of hematopoietic cells but bear appropriate microenvironments allowing lymphoid cell generation. Therefore, mice with severe combined immunodeficiency (SCID), lacking only mature B and T lineage cells, are excellent recipients for such studies (Bosma *et al.*, 1983).

Early B lineage cells in bone marrow can be defined by coexpression of B220 and leukosialin (CD43) (Hardy *et al.*, 1991). Cells expressing B220, CD43, and the heat-stable antigen (HSA) were shown to possess largely D–J, but not V–DJ, rearrangements and thus consist of an enriched population of "pro-B cells." Transfer of such pro-B fractions sorted from bone marrow and fetal liver reconstituted B cells with distinct phenotypes: fetal pro-B gave rise to B cells with low levels of IgD, many expressing CD5, whereas adult pro-B gave rise to B cells with high levels of IgD, few expressing CD5 (Hardy and Hayakawa, 1991). This result provides strong evidence that CD5 B cells are the progeny of a distinct fetal B cell differentiation pathway. In addition, data also suggest that the progeny of fetal pro-B cells do not all bear CD5, although most are $IgD^{\pm}$, as with CD5 B cells.

Another approach for enriching distinct progenitors is to determine a distinct anatomical location for CD5 B cell precursors, and the work of Kearney and co-workers (Solvason *et al.*, 1991) suggests that the fetal omentum is such an area. They engrafted total fetal omentum into SCID mice and then observed restricted repopulation of CD5 B cells (along with IgD^-CD5^- B cells). T cell were not produced, which suggests that the fetal omentum is a site of B lineage cells committed to a distinct differentiation pathway that does not yield "conventional" B cells. The human fetal omentum is also a site of B lymphopoiesis (Solvason and Kearney, 1992). Whether such omentum B progenitor cells are disseminated from fetal liver remains to be determined.

The transfer data described previously all employed adult recipi-

ents, either SCID or irradiated mice. Whether a stem cell (and at what stage) from adult animals might give rise to CD5 B cells on transfer into a fetal or neonatal environment is an interesting but unsettled issue. Nevertheless, fetal and adult B lineage cells *in vivo* appear to be already committed to the differential levels of CD5 induction, regardless of environment since CD5 expression was clearly seen after short-term culture of fetal pro-B cells, but not adult pro-B cells, and both cultures yielded similar numbers of IgM^+ cells. A potential distinct precursor for CD5 B cells was also suggested by earlier work subsetting the $lineage^-$ (Lin^-) fraction (the 2–5% of cells lacking surface expression characteristic of B, T, myeloid, or erythroid lineages) of newborn liver with Thy-1 and asialo-GM_1 (aGM_1), showing that one fraction gave rise to CD5 B cells when cultured on a fetal liver-derived stromal line (Hardy *et al.*, 1987b). Thus, whereas Thy-$1^+aGM_1^-$ cells proliferated most, they generated few $B220^+$ cells in short-term culture, whereas Thy-$1^-aGM_1^+$ cells proliferated to a more limited extent, but most (greater than 90%) began to express B220 and a large fraction (up to 50%) became surface IgM^+ after 2 weeks in culture. Many of these IgM^+ cells also expressed CD5. A cell population with this Lin^-Thy-$1^-aGM_1^+$ phenotype was found in fetal and newborn liver, and in bone marrow of young mice, but few were detectable in adult bone marrow (Hardy *et al.*, 1987b), suggesting that aGM_1 expression may distinguish between fetal and adult early B lineage precursor cells.

CD5 B cells can self-reconstitute a population of CD5 B cells when injected into irradiated recipients (Hayakawa *et al.*, 1986b). Furthermore, this reconstituted population remains at stable levels in recipients for a long period (6 months or more) (Hayakawa *et al.*, 1986b). Experiments that involved injecting peritoneal cavity B cells into newborn mice demonstrated significant proliferation of CD5 B cells by the time that the recipients had reached adulthood (Forster and Rajewsky, 1987). This repopulating capacity is not limited to CD5 B cells in the peritoneal cavity because splenic Ig^+ cells in the newborn (including $CD5^+$ B cells) also reconstitute exclusively CD5 B cells (Hayakawa *et al.*, 1986a,b). Whether this self-renewal activity can be applied to all CD5 B cells newly generated in early ontogeny, or is instead a feature of those CD5 B cells selectively retained into adulthood, is not established. Nevertheless, this observation provides a simple explanation for how CD5 B cells can maintain stable numbers through life in the absence of constant resupply from the bone marrow.

Lalor *et al.* (1989a,b) further characterized this property by utilizing the surface immunoglobulin phenotype of CD5 B cells. Treatment of

immunoglobulin allotype homozygous mice with anti-IgM specific for this allotype completely abrogated the generation of CD5 B cells in later life, whereas other ($CD5^-$) B cells of this allotype appeared after cessation of antibody administration. Total lymphoid irradiation of adult animals can also eliminate the CD5 B cell population, whereas the $CD5^-IgD^{++}$ B cells and the T cells will regenerate (E. Field, R. Hardy, and K. Hayakawa, 1989, unpublished). Thus eliminating or blocking the generation of CD5 B cells during an early developmental stage (or eliminating them from the adult) appears to eliminate such cells for the life of the animal in most cases, but some have observed eventual regeneration (Farinas *et al.*, 1990; Ishida *et al.*, 1992). Thus, the possibility that there may be a reservoir of precursors for fetal B cells that is maintained through life cannot be excluded. In this view, as Lalor *et al.* (1989) propose, continuing $CD5^+$ B cell generation from such precursors might be normally blocked by feedback regulation.

IV. Model for Generation of CD5 B Cells

The data described above suggest a model of B cell generation that regards fetal and adult B cell differentiation as significantly different (Fig. 1). The potential to generate CD5 B cells appears largely restricted to early ontogeny. Furthermore, the B lineage cells generated during this early developmental timing *in vivo* appear to be committed to a unique fetal B cell pathway, the majority of which begin to express CD5 and which do not progress into a stable IgD^{++} B cell population (in mice). At least some of these CD5 B cells can persist (or maintain their progeny) for the lifetime of the animal, as shown by self-renewing activity. Indeed, the CD5 B cell progenies of fetal day 16 gestation pro-B cells have been observed in the absence of any other lymphoid cells for more than 1 year (R. Hardy and K. Hayakawa, 1992, unpublished). However, the extent of cell dynamics within the fetal B cell population is not yet established. As described later, an alteration of repertoire in the CD5 B cell population with time suggests selection by antigens, likely positive selection by self-antigens. Furthermore, since bone marrow in young animals still retains some capacity to generate CD5 B cells (although fewer) in cell transfer experiments, its contribution to the adult CD5 B cell pool in unmanipulated animals cannot be completely excluded. However, experiments that block CD5 B cell generation in neonates or eliminate it from adults strongly suggests that the majority of the CD5 B cell subset in adult mice is derived during the fetal (to early postnatal) stage of ontogeny.

Since stem cell-enriched or B lineage-committed fractions in the

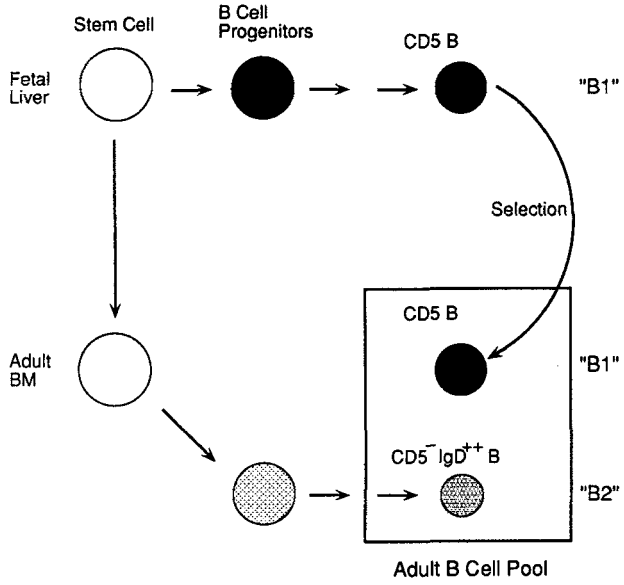

FIG. 1. Developmental switch in B lymphopoiesis: model for the developmentally regulated generation of B cell precursors. In this view, cells at the pro-B cell stage and after are readily distinguishable. The hematopoietic stem cell population has also been shown as distinct, but this is not absolutely necessary.

bone marrow of adult mice (>3 months) do not generate CD5 B cells, this supports the concept of a "developmental switch" in B lymphopoiesis. Some have proposed to refer to CD5 cells as "B-la" cells and to those $CD5^-$ B cells that share some phenotypic and functional features as "B-1b" cells (Kantor, 1991). This is intended to contrast these subsets, regardless of CD5 expression, with the predominant "conventional" population of $CD5^-IgD^{++}$ B cells in spleen, which might be termed B-2 cells in this nomenclature. However, this terminology implies a relationship between B-1a and B-1b cells that is not established and that can lead to confusion. Thus, some regard generation of $IgD^{\pm}Mac\text{-}1^+$ cells ("B-1b") in the peritoneal cavity as evidence of a "CD5 B cell type" repopulation because of the location and the self-reconstituting activity. But it is clear that both fetal and adult B lineage-committed fractions can contribute to this pool (Hardy and Hayakawa, 1991; Kantor *et al.*, 1992), and so the definition of B-1b as a distinct "lineage" becomes rather murky. In the model we propose, it may be more appropriate simply to term fetally generated B cells as B-1 cells, and B cells generated later in ontogeny (in the adult) ad B-2 cells.

It is possible that the $CD5^-IgD\pm$ cells produced in ontogeny (perhaps even derived from $CD5^-IgD^{++}$ cells after antigen exposure) may share a number of similarities with fetally derived CD5 B cells. However, the differentiation and diversification of the progeny of bone marrow B lineage cells in the periphery are still not completely understood (for example, the origins of the marginal zone cells), and so further studies are clearly required. For this reason, in this review, we focused our attention on $CD5^+$ B cells, even though this may overlook some closely related $CD5^-$ B cells derived from the same fetal precursor source.

V. Properties of Murine CD5 B Cells

A. Surface Phenotype

CD5 B cells sorted from NZB spleen or BALB/c peritoneal cavity show lymphoid morphology (large nucleus and scanty cytoplasm) (Hayakawa *et al.*, 1983) and express many surface molecules common to B cells (Hayakawa *et al.*, 1983). However, compared with small (high IgD expressing) B cells, they are relatively larger, show more internal granularity (measured by large-angle light scatter on the cell sorter) (Hayakawa *et al.*, 1986a), and are slightly more adherent to glass (Hayakawa *et al.*, 1986a), but are smaller than mitogen-activated B cells and less granular or adherent than myeloid cells. CD5 B cells express different levels of IgM, depending on strains of mice or age, and somewhat higher average levels of surface IgM were found in BALB/c and NZB mice. As described earlier, regardless of strains or ages, CD5 B cells consistently show much lower levels of IgD than do typical ($CD5^-$) B cells (Hayakawa *et al.*, 1983, 1986a). In contrast, the IgD level does not distinguish between human $CD5^+$ and $CD5^-$ B cells (Antin *et al.*, 1986). One of the high-molecular-weight CD45 isoforms (B220) detected by the monoclonal antibody RA3-6B2 is distinctively lower compared with most splenic $CD5^-$ B cells (Herzenberg *et al.*, 1986). Mac-1 (CD11b), a molecule normally considered to be characteristic of macrophages, is also detectable at low levels on CD5 B cells in the peritoneal cavity (Herzenberg *et al.*, 1986; de la Hera *et al.*, 1987), but not on CD5 B cells in spleen. CD5 is the only one of a group of common T cell differentiation antigens (in both mice and humans) found on a subset of B cells and at a distinctive low level: 10 times lower compared with the average expression found on T cells (Hayakawa *et al.*, 1983, 1984a; Bofill *et al.*, 1985; Hardy *et al.*, 1987a). MHC class II molecules are expressed on CD5 B cells similar to expression on $CD5^-$ B cells (Hayakawa *et al.*, 1983).

TABLE II

FEATURES ASSOCIATED WITH CD5 B CELLS IN DIFFERENT MOUSE STRAINS

	CD5 B cell[a]			Serum IgM			
Mouse strain	Frequency	Proliferation	Malignancy	Total	Autoantibody	Autoimmune Disease	Comments
NZB	+++	+	+	++	++	++	SLE autoimmune syndrome; lymphoma
Me^v	+++	n.a.	n.a.	++	++	++	Severe autoimmunity
SM/J	++	+	−	+	+	−	Autoantibody without obvious disease
BALB/c	+	±	±	+	±	−	Clonal CD5 B cell proliferation in aged animals
B6	+	−	−	+	±	−	
SJ/L	±	−	−	+	±	−	
RIIIS/J	−	−	−	±	−	−	Low responder to PC
CBA/N	−[b]	−	−	±	−	−	xid blocks autoimmune disease in cross to NZB

[a] n.a., not applicable due to early death.

[b] CD5 B cells detectable in spleen, but not peritoneal cavity.

A number of molecules found at distinctive levels are often considered to reflect an "activated" state (Table III). Among these are elevated Pgp-1 (Ly24), diminished CD23 (low-affinity receptor for IgE), increased HSA (initially reported as 10.1), and detectable IL-5 receptor (Murphy *et al.*, 1990; Waldschmidt *et al.*, 1988; Hitoshi *et al.*, 1990). This might be interpreted as generally supporting the view that CD5 B cells are an "activated population" or even that CD5 is yet another activation marker for B cells, as some have suggested (Cong *et al.*, 1991). However, Rothstein and co-workers pointed out that the expression of other surface molecules, notably Ia, distinguishes these cells from typical mitogen-activated B cells (Murphy *et al.*, 1990). Furthermore, the expression of several of these molecules is not notably different on CD5 B cells from germ-free mice (Murphy *et al.*, 1990), suggesting that external antigens are not responsible for the generation or distinctive phenotype of CD5 B cells.

B. Strain Variation in Levels of CD5 B Cells

A survey of "normal" inbred mouse strains revealed genetic influences on the level of CD5 B cells in adult peritoneal cavity, wherein each strain shows its own stable level (Hayakawa *et al.*, 1986a) (Table II). BALB/c and their H-2 or immunoglobulin allotype (Igh) congenic strains have relatively high levels compared with others (C57BL/10, CBA, A.TH, 129/Sv, etc.) (Hayakawa *et al.*, 1986), whereas SJL and the Igh congenic SJA strain both show a distinctively lower level (Hayakawa *et al.*, 1984a, 1986a,b). The RIIIS/J strain is reported to lack CD5 B cells, but $CD5^{-}IgD^{\pm}$ cells are found at normal levels in the peritoneal cavity (Hiernaux *et al.*, 1989). The autoimmune mouse strain NZB shows a distinctively higher level of CD5 B cells compared with all normal strains by 2 months of age. Another defective strain, "moth-eaten viable" (me^{v}), carries a recessive single gene defect, resulting in severe immunodeficiency together with autoimmunity (Schultz *et al.*, 1984), and has decreased numbers of B cells that are almost exclusively $CD5^{+}$ (Sidman *et al.*, 1986). These mice die early before the development of other types of B cells would be expected. Finally, mice bearing the X chromosome-linked immunodeficiency, xid (Scher, 1982), have impaired CD5 B cell generation: CD5 B cells are absent from the peritoneal cavity and autoantibody specificities characteristic of CD5 B cells are lacking (Hayakawa *et al.*, 1986a,b). NZB mice congenic for the *xid* gene (which eliminates the autoimmune condition of this strain) also show this deficit (Hayakawa *et al.*, 1983).

TABLE III
CELL SURFACE PHENOTYPES OF FETAL AND ADULT-DERIVED B CELL SUBSETS IN ADULT MICE[a]

B cell subset	IgM	IgD	CD5	Mac-1 (CD11b)	CD23	Pgp-1 (CD44)	HSA	CD43	B220 (6B2 epitope)
Fetal ("B1")	++	±	+ (some −)	+ (PerC), − (spl)	−	++	++	+	+
Adult ("B2")	+	++ (some ±)	−	−	+	+	+	−	++

[a] Predominant phenotype of cells in each population. Some cells in each fraction may show an exceptional phenotype, as, for example, the "B-1b" cells generated from bone marrow and the marginal zone B cells. PerC, peritoneal cavity cells; spl, spleen cells.

C. DIFFERENTIATION AND ISOTYPE EXPRESSION

Almost all $CD5^+$ B lineage cells are found to express surface immunoglobulin and few (if any) cells can be defined in situ as $CD5^+$ pre- or pro-B cells (Hardy *et al.*, 1984); the B lineage cell lines transformed by retroviruses are a notable exception (Holmes *et al.*, 1986a). Most CD5 B cells express IgM (with low to undetectable levels of IgD), although IgG expression was found on some peritoneal cavity CD5 B cells in association with IgM (R. Hardy and K. Hayakawa, unpublished). CD5 B cells can produce IgG as shown by cell transfer experiments (Herzenberg *et al.*, 1986; Solvason *et al.*, 1991) and LPS stimulation *in vitro* (authors, 1988, unpublished). There is no clear-cut evidence at present whether CD5 B cells terminally switch to expression of IgG alone (after deleting μ) or whether instead these cells express other isotypes by transcriptional isotype regulation (Chen *et al.*, 1986; Braun *et al.*, 1986). Furthermore, the capacity of this population to undergo somatic mutation is not clear.

This absence of IgG^+ (IgM^-) $CD5^+$ B cells may be explained by the loss of CD5 expression on terminal differentiation. Data from Kroese and collaborators investigating the origins of the IgA plasma cells in gut tissue suggested unexpectedly that many of these cells are derived from CD5 B cells (Kroese *et al.*, 1989). They generated chimeric mice in which bone marrow-derived B cells and CD5 B cells differed in immunoglobulin allotype. They then analyzed the Ig allotypes of gut-

associated tissues, finding that whereas most Peyer's patch surface IgM^+ cells were bone marrow derived (similar to spleen or lymph nodes), one-half or more of the IgM and IgA plasma cells in the lamina propria were of the CD5 B cell allotype, indicating that CD5 B cells may play an important role in the mucosal immune system.

An increased frequency of λ light chain expression (10–20%) in the normal mouse CD5 B cell population was found in peritoneal cavity CD5 B cells (Hayakawa *et al.*, 1986a,b) in the adult. Furthermore, $CD5^+$ cell lines spontaneously arising from spleen tissue frequently expressed λ light chain (Braun, 1983). In addition, there is an increased level of λ-bearing immunoglobulin in the sera of autoimmune "moth-eaten" mice (Schultz and Greene, 1976; Sidman *et al.*, 1986), wherein most B cells are $CD5^+$ (Sidman *et al.*, 1986). In the $CD5^+$ NFS-5 line, rearrangement of λ light chain in the presence of a productive κ rearrangement has been demonstrated (Hardy *et al.*, 1986), suggesting a possible accumulation of λ-expressing cells over time. This line also showed V_H gene replacement in subclones (Kleinfield *et al.*, 1986). Such differences in immunoglobulin gene regulation between CD5 B cells and conventional B cells could reflect some of the (potentially) numerous differences in the differentiation program of these cells. However, at present there is no evidence that such modes of differentiation are particularly favored by normal CD5 B cells.

D. Cytokine Production and Regulation

B cells could exhibit distinctive functions by secreting novel cytokines, affecting other cell types or even regulating their own growth in an autocrine fashion. $CD5^+$ B lymphoma cell lines have been shown to produce several lymphokines, (O'Garra *et al.*, 1990), including TGF-β, IL-10, IL-6, TNF-α, TNF-β, and G-CSF. Among these, IL-10 was confirmed to be produced preferentially by CD5 B cells in normal mice. O'Garra, Howard, and collaborators investigated cytokine production by sorted B cells subsets using PCR (O'Garra *et al.*, 1990, 1992). They found that $CD5^+$ B cell lines and even normal sorted populations differed in their production of IL-10 (with or without LPS-induced stimulation): production of this cytokine was enriched in the normal CD5 B cell subset. More recently, this same group demonstrated that continuous treatment with anti-IL-10 from birth eliminated the CD5 B cell population from the peritoneal cavity (Ishida *et al.*, 1992). The effect was subsequently interpreted as an indirect effect, in that anti-IL-10 treatment resulted in increased levels of IFN-γ, which appeared directly responsible for the decreased numbers of CD5 B cells. Therefore, the original idea of IL-10 as an important

autocrine factor for CD5 B cells is not clear. However, since IL-10 is known to be associated with helper T cell activity, its preferential production by CD5 B cells suggests that some interesting regulatory functions in the antibody response could be mediated by this population.

With regard to cytokine receptors, it has also been reported by two groups that CD5 B cells are highly responsive to IL-5 (Wetzel, 1989; Umland *et al.*, 1989), although this is also true of IgD^{++} B cells if they are first activated with anti-IgM (Allison *et al.*, 1991). An antibody to IL-5 receptor stains most CD5 B cells (Hitoshi *et al.*, 1990).

E. CD5 Expression on Murine B Cell Malignancies

Abnormal CD5 B cell proliferation occurs in NZB mice and is elevated in (NZB × NZW)F_1 mice (Hayakawa *et al.*, 1983). Old BALB/c mice also occasionally show a distinctively expanded population of splenic CD5 B cells (Stall *et al.*, 1988). In particular, pauciclonal proliferation of CD5 B cells occurs frequently in NZB mice, is extreme in the peritoneal cavity, and often occurs later in the spleen. Such proliferation results in splenomegaly consisting almost entirely of CD5 B cells, and their presence even in lymph nodes suggests expansion into most lymphoid compartments. Hyperdiploid cells, which represent a premalignant stage (Raveche *et al.*, 1981), reside in the NZB peritoneal cavity of the CD5 B population (Seldin *et al.*, 1987; Raveche *et al.*, 1988).

An extensive series of studies on the CH B lymphomas has been carried out by Haughton and collaborators (1986). This murine B lymphoma system allows one to obtain a set of cell lines generated independently *in vivo* and carries significant implications on the association of CD5 B cells with B lymphoma. The CH lymphomas arose in the spleen of a particular double-congenic mouse strain, B10.H-2^aH-4^b, either spontaneously or after transfer of spleen cells (SRBC hyperimmunized) into syngeneic or F_1 recipients (Lanier *et al.*, 1982). All (of 27) B lymphomas express IgM and surprisingly all express CD5 at various levels (Pennell *et al.*, 1985; Haughton *et al.*, 1986). Moreover, although they were elicited independently, many shared idiotopes, and analysis of a large group suggested an interconnected family of related specificities. Thus, seven antiidiotype antisera to individual lymphomas were sufficient to define a series of 12 shared idiotopes expressed by 21 of the 27 CH tumors (Pennell *et al.*, 1985; Bishop and Haughton, 1985).

Inbred Swiss strain NFS mice are lacking for ecotropic murine leukemia virus (MuLV) and show a low incidence of nonthymic tumors.

NFS mice congenic for MuLV (NFS/N v-congenic) show a more than threefold increase of nonthymic lymphomas (predominantly B lineage) and myelogenous leukemias by 18 months of age (Fredrickson *et al.*, 1984). Davidson *et al.* (1984) found that almost all B lymphomas occurring in NFS/N v-congenic mice show CD5 expression. Interestingly, Holmes *et al.* (1986b) reported that a majority of lines transformed by various retroviruses (containing the *fes*, *ras*, and *src* oncogenes) expressed CD5. These cell lines have pro- or pre-B cell phenotypes, as determined by Ig gene rearrangement and surface phenotype. Interestingly, some had shared pre-B/myeloid characteristics and actually differentiated into macrophages (Davidson *et al.*, 1988; Principato *et al.*, 1990).

CD5 B cells also show unique characteristics in culture: whereas most B cells from peripheral lymphoid organs die after a short time in culture, CD5 B cells show prolonged survival (Hayakawa *et al.*, 1983). This is clear in NZB CD5 B cells (in both the spleen and peritoneal cavity) and also in BALB/c CD5 B cells in the peritoneal cavity. Braun found that the IgM^+ B cells surviving after the crisis phase of culture all expressed low levels of CD5 (Braun, 1983). Such lines were generated from several different strains and grew rapidly after the initial phase. Further analysis showed that these lines had hyperexpression and genomic amplification of c-*myc* (Citri *et al.*, 1987), features not characteristic of the normal CD5 B cell population.

VI. Biases in Specificity

One of the earliest findings characteristic of CD5 B cells was their association with self-reactivity. CD5 B cells were first detected as a B cell subset in the autoimmune mouse strain NZB (Manohar *et al.*, 1982; Hardy *et al.*, 1982; Hayakawa *et al.*, 1983). In addition, $CD5^+$ B cell neoplasias have been found to show a high frequency of autoreactivity (Haughton *et al.*, 1986; Kipps *et al.*, 1988; Sthoeger *et al.*, 1989). Finally, certain types of germ-line-encoded IgM autoantibody, such as that reacting with bromelain-treated mouse red blood cells (BrMRBC) or thymocytes have been shown to derive preferentially from CD5 B cells (Hayakawa *et al.*, 1984b, 1990; Hardy *et al.*, 1989). IgM autoantibody naturally found in normal mouse serum ("natural autoantibody") appears to derive preferentially from CD5 B cells, although whether this is true of all natural autoantibodies is not clear. These antibodies, enriched in CD5 B cells, anti-BrMRBC, and antithymocytes, exhibit specific binding activity and are not "polyspecific" (Conger *et al.*, 1992; Hayakawa *et al.*, 1990, 1992), a feature claimed to

characterize many antibodies produced by CD5 B cells in mice and humans (Kearney *et al.*, 1992; Casali *et al.*, 1988). Since the clear definition of "polyspecificity" is a controversial issue and requires more refinement, we will not attempt to address it further. One final speculation on this topic is that many of the determinants recognized by CD5 B cells are carbohydrates on glycoproteins or gycolipids that could be shared among different molecules, and indeed an example is illustrated by some antithymocyte antibodies (Hayakawa *et al.*, 1990).

In the following section we review some of the self-reactivity associated with CD5 B cells and mention several possible explanations for the significance of such reactivity. The cross-reactivity by anti-BrMRBC with a component of bacterial cell wall has been offered as a possible explanation for the enrichment of this specificity (Mercolino *et al.*, 1986), although the presence of this specificity in germ-free mice argues against this as a causative agent (Cunningham, 1974). Alternatively, some have suggested that clearance of senescent cells or denatured proteins might be a function for natural autoantibodies (Boyden, 1964), which several of these specificities represent. Perhaps the most appealing suggestion is that the V genes encoding natural autoantibodies have been selected into the germ-line because such specificities cross-react with determinants present on frequently encountered pathogens. In this model, the enrichment of the specificities in CD5 B cells due to self-reactivity would then confer a natural immunity to these pathogens. This may be particularly important during the neonatal stage after immunity due to maternal antibodies has decayed but before the individual's own T-dependent immune system has developed.

A. Autoimmune Mice: NZB, Motheaten, and Lymphoproliferation Defect

NZB spleen cells secrete high levels of IgM, including autoantibody (anti-ssDNA, anti-T cell)) when incubated *in vitro*. This "spontaneous" secretion is often solely from CF5 B cells (Hayakawa *et al.*, 1983), and in a study by Hayakawa *et al.* (1984b), the autoantibody ratio (to total secreted IgM) was enriched in CD5 B cell-derived IgM. Since the total serum IgM level and splenic CD5 B cell level roughly correlate in young NZB adults (A. Steinberg, R. Hardy, and K. Hayakawa, 1983, unpublished), the CD5 B cell population in NZB mice may contain a higher frequency of Ig-secreting cells than other B cells and may be largely responsible for autoantibody secretion, in particular of the IgM isotype. However, because NZB IgM secretion appears affected by two genetic factors, one affecting the number of IgM-

secreting cells and another the amount secreted per cell (Manny *et al.*, 1979), the presence of CD5 B cells in increased numbers must be considered separately from their etiologic significance in autoantibody secretion. In fact, old NZB mice eventually reveal large deviations in the level of $CD5^+$ B cells, some showing pauciclonal proliferation without Ig secretion. Nevertheless, although the study of the immune system in NZB mice involves several genetic traits unique to this strain, examination of CD5 B cells in NZB mice clearly demonstrates two distinctive capabilities of this population, namely proliferation and high autoantibody secretion.

Motheaten (*me*) mice also serve to implicate CD5 B cells with autoantibody. These *me* mice die of severe immune complex syndrome shortly after birth (Schultz and Greene, 1976). Abnormally high levels of serum autoantibodies found in such *me* or *me viable* (me^v) (Schultz *et al.*, 1984; Schultz and Greene, 1976) mice must be strongly related to CD5 B cells since almost all B cells in me^v mice show CD5 expression (Sidman *et al.*, 1986). A series of studies by Sidman and collaborators (1984) showed that such high immunoglobulin secretion seems to be promoted by B cell-derived B cell maturation factors uniquely elevated in *me* mice. Growth and/or differentiation factor secretion from human $CD5^+$ B cell lines (Kawamura *et al.*, 1986) and by murine CD5 B cell-derived hybridomas (Sherr *et al.*, 1987) has also been suggested. It is tempting to invoke secretion of such factors as due to unique CD5 B cell characteristics; however, we must admit that it remains unclear whether secretion of such factor(s) is directly responsible for the unusual properties of CD5 B cells (as distinguished from other B cells). Sidman and collaborators have published an extensive study of V gene usage in autoreactive hybridomas established from this strain (Kasturi *et al.*, 1990), suggesting that they are germ-line encoded.

Mice with the *lpr* defect have a single gene mutation that results in the lymphoproliferation of T linage cells of an unusual phenotype and a lupus-like autoimmune syndrome (Cohen and Eisenberg, 1992). These mice do not show an increased frequency of CD5 B cells (Hayakawa *et al.*, 1983) and the autoantibodies in this strain are predominantly of the IgG isotype, in contrast with NZB and motheaten. Shlomchik and Weigert have shown that the Ig V gene sequences that encode such autoantibodies are mutated in a manner similar to somatic hypermutation during affinity maturation in an immune response (Shlomchik *et al.*, 1987). Eisenberg and co-workers investigated the origins of these autoantibodies in *lpr* mice by analyzing allotype chimeric mice, wherein bone marrow and CD5 B cells are distinguishable (Reap *et al.*, 1993). They found that autoantibodies in this strain were produced

largely by the conventional (bone marrow derived) B cells, not by CD5 B cells. Thus, while CD5 B cell are clearly responsible for production of many "natural" autoantibodies (and autoantibodies in certain autoimmune strains), "pathogenic" autoantibodies, characterized by somatic hypermutation, are likely produced by conventional B cells, probably with the help of T cells.

B. Anti-BrMRBC/Anti-PtC

Normal mice contain a surprisingly large number of Ig-secreting cells, enriched in the peritoneal cavity, that can lyse mouse erythrocytes previously treated with the proteolytic enzyme bromelain (Bussard, 1966; Pages and Bussard, 1975; Steele and Cunningham, 1978; Lord and Dutton, 1975). Deliberate antigen (BrMRBC) immunization does not increase their numbers so there is no memory response (Cunningham, 1974). Their frequency can be increased by polyclonal activation (Cunningham, 1974; Hammarstrom *et al.*, 1976; Hang *et al.*, 1983), but cells with this specificity are also present in germ-free mice (Cunningham, 1974) and thus are not simply the result of stimulation by gut flora. Their function is unknown, but some have hypothesized a role in the clearance of senescent erythrocytes, although this is controversial (Kaushik *et al.*, 1988).

A difference in the antibody repertoires of CD5 B cells and $CD5^{-}IgD^{++}$ B cells was first suggested by the restriction of anti-BrMRBC autoantibody secretion to CD5 B cells (Hayakawa *et al.*, 1984b). In these early experiments, essentially all of the plaque-forming cells (PFC) in the spleen that lysed BrMRBC were found in the fraction sorted as $IgM^{+}CD5^{+}$ in BALB/c mice. Due to the paucity of splenic CD5 B cells in this mouse strain and low CD5 expression, the purity of the sorted cells was less than in NZB. However, this enrichment appeared specific since the anti-BrMRBC PFCs did not stain with an isotype-matched control antibody and few PFCs were obtained from the $CD5^{-}$ B cell fraction that comprised most of the splenic B cells. Subsequently, it was found that anti-BrMRBC-secreting cells in the peritoneal cavity, long known as a location curiously enriched for such antibody-secreting cells, were accounted for by an enrichment of CD5 B cells (Hayakawa *et al.*, 1986a). Thus, the association between CD5 B cells and anti-BrMRBC PFC has been observed in most strains. However, in certain strains, such an CBA, a significant portion of anti-BrMRBC PFC derives from $CD5^{-}IgD^{\pm}$ B cells in the peritoneal cavity (in addition to the CD5 B cells) (A. M. Stall, personal communication; Andrew *et al.*, 1990). This may be explained by the observation that not all fetal-derived B cells express

CD5 (assuming that most of this specificity in adult B cells comes from enrichment in the fetal B cell population); furthermore, it shows that there is not an absolute correlation between CD5 expression and the anti-BrMRBC specificity. Perhaps most definitively, analysis of mice reconstituted with bone marrow precursors showed few cells with this specificity (Hayakawa *et al.*, 1985).

Haughton's group utilized an assay with fluorochrome-loaded vesicles that included phosphatidylcholine (PtC) in their membrane to show that a significant number of peritoneal cells shared reactivity with several of their CH lymphomas known to bind BrMRBC (Mercolino *et al.*, 1986, 1988). They also noted in this study that the frequency of PtC-binding B cells appeared to increase in the peritoneal cavity between 2 weeks and several months of age, suggesting a gradual accumulation of cells with this specificity. By sorting PtC-staining cells from the peritoneal cavity, they found that all anti-BrMRBC-secreting cells were contained in this population (Mercolino *et al.*, 1988).

It is possible to screen for the BrMRBC reactivity in serum or culture supernatants by fluorescence analysis to detect IgM bound to bromelain-treated erythrocytes. As with earlier PFC experiments, results showed that depletion of CD5 B cells from the spleen specifically eliminated production of this autoantibody and, conversely, that purified CD5 B cells uniquely exhibited anti-BrMRBC secretion elicited by LPS (Hardy *et al.*, 1989). The enrichment of BrMRBC-specific cells within the CD5 B cell population has also been demonstrated by analyzing hybridomas made from SM/J mice (wherein the frequency of CD5 B cells was 3–5% in spleen, which facilitated pure cell sorting). Anti-BrMRBC-producing hybridoma clones composed 1–2% of the total hybridomas generated from sorted CD5 B cells, but only 0.03% in hybridomas made from sorted $CD5^{-}IgD^{++}$ B cells (Hardy *et al.*, 1989). These CD5 B cell-derived hybridomas producing anti-BrMRBC antibodies were mostly $V_H11/V_\kappa9$ (80%) and the rest were $V_H12/V_\kappa4$. The single hybridoma obtained from $CD5^{-}IgD^{++}$ cells that showed anti-BrMRBC activity was found to utilize an S107 V_H. Analysis of the binding of these hybridoma-derived antibodies revealed differences in fine specificty between V_H11- and V_H12-encoded immunoglobulins (Hardy *et al.*, 1989). Conger, Corley and co-workers have studied this difference in detail (Conger *et al.*, 1991). The issue of V gene usage is covered in Section VII.

C. Antithymocyte Autoantibody

The production of antibodies reactive to the determinants present on intact thymocytes occurs spontaneously in normal animals (Shirai and Mellors, 1971; Martin and Martin, 1975; Eisenberg *et al.*, 1979), and

although high levels of antithymocyte autoantibody (ATA) have been found in animals with autoimmune disease (e.g., NZB mice), high levels have also been detected in some strains of mice lacking any obvious pathology (SM/J mice; Eisenberg, *et al.*, 1979). Both NZB and SM/J mice have elevated levels of CD5 B cells (Hayakawa *et al.*, 1983; Rabinovitch *et al.*, 1986) along with increased levels of antithymocyte autoantibodies in their sera, suggesting an association. Furthermore, CD5 B cells in NZB mice are largely responsible for the spontaneous secretion of IgM *in vitro*, including ATA (Hayakawa *et al.*, 1984b). However, whether this is due to the unique predisposition of NZB CD5 B cells to secrete immunoglobulin spontaneously *in vitro* or instead to a higher frequency of ATA-specific B cells in the CD5 B cell population is not clear.

Analysis of LPS-activated supernatants of cultures containing sorted $CD5^+$ B cells or $CD5^-IgD^{++}$ B cells and also cultures of cell fractions specifically depleted of $CD5^+$ B cells or $CD5^-$ B cells show a clear correlation between ATA production and CD5 B cells (Hayakawa *et al.*, 1990). These data strongly suggest that CD5 B cells are responsible for natural ATA secretion in most strains and, further, that the increased population of CD5 B cells in SM/J mice is responsible for the characteristically high levels of serum ATA found in this mouse strain. ATA production by CD5 B cells was characterized further by establishing hybridomas from CD5 B cells and $CD5^-IgD^{++}$ B cells sorted from a pool of SM/J mouse spleen (Hayakawa *et al.*, 1988). Initial screening of 576 IgM-secreting wells from both CD5 B cells and $CD5^-IgD^{++}$ B cell fusions showed that the frequency of clones with antithymocyte-binding reactivity was at least 10-fold higher in the CD5 B cell subset, consistent with results from the ATA secretion assay. Analysis of these hybridoma clones revealed several distinct ATA binding profiles (Hayakawa *et al.*, 1990). Most monoclonal IgM ATAs from CD5 B cells selected for thymocyte-binding activity showed specific binding to T lineage cells and a few showed that anti-B/T cell reactivity found with perinatal B cell hybridomas (Lehuen *et al.*, 1992). Furthermore, diverse fine specificities among ATAs were evident, with differential reactivity to thymocytes at various maturational stages, variable binding to peripheral T cells ($CD4^+$ and/or $CD8^+$), and different degrees of species cross-reactivity (mouse/rat).

The reactivity of 11 of these autoantibodies was sensitive to prior treatment of target cells with sodium metaperiodate, which preferentially oxidizes terminal sialic acid groups, implicating carbohydrate as an important constituent of the antigenic determinant (Hayakawa *et al.*, 1990). Furthermore, a large fraction were found to be carbohydrate determinants whose expression depended on the Thy-1-molecule, ei-

ther residing on the Thy-1 glycoprotein or on a molecule that is in close proximity. Another antibody reacted with a determinant that was carbohyrate in nature but was expressed on a particular glycolipid, a ganglioside (Greer *et al.*, 1993). Anti-glycolipid activity was also seen with other ATAs (P. Mühlradt and K. Hayakawa, 1993, unpublished).

Lehuen and co-workers (1992), reported similar findings for a panel of perinatal hybridomas, but suggested that several of the antibodies that were reacting with a phospholipase-sensitive determinant were recognizing an unidentified protein of 100 kDa molecular mass. They suggested that such antilymphocyte reactivity by B cells in young mice could be important in the development of the normal adult lymphoid compartment.

D. Other Specificities: Anticarbohydrate and Antiphosphorylcholine

Several other specificities have been suggested as deriving all or in part from CD5 B cells. Besides anti-T cell reactivity, anti-B cell-specific cells were found to be enriched in the CD5 B cell subset (Hayakawa *et al.*, 1992). Most these determinants appear to be of a carbohydrate nature, although confirmation of this awaits further study. If confirmed, this will not be a novel finding, since distinctively branched carbohydrate structures have been shown previously to mark particular differentiation stages or cell types (Feizi, 1985). Forster and Rajewsky (1987) ascribed anti-1,3-dextran reactivity to peritoneal CD5 B cells identified using a neonatal cell transfer system. More recently, Cazenave's group demonstrated an enrichment of $T15^+$ antiphosphorylcholine (anti-PC) reactivity in CD5 B cells (Masmoudi *et al.*, 1990), which had been suggested previously (Herzenberg *et al.*, 1986). They showed that the T15 idiotype component of anti-PC was largely derived from CD5 B cells, but that non-T15 anti-PC could be elicited from $CD5^-$ B cells. Another group reported that the λ-restricted anti-O/core (but not anit-lipid A) response to LPS derived from CD5 cells (Su *et al.*, 1991), supporting a role for these cells in a primary response to common bacterial pathogens.

E. Response to Foreign Antigens

Because of the rarity of CD5 B cells in normal adult spleen, it is difficult to ascertain their ability to respond to typical "laboratory" antigens simply by cell-sorting purification. The increased CD5 B population in NZB provided an opportunity to purify splenic CD5 B cells by fluorescence-activated cell sorting (FACS) sufficiently to eliminate other contaminating B cells, and to test this question directly

(Hayakawa *et al.*, 1984b). Studies on these B cells in me^v mice also provide information on CD5$^+$ B cell function, since most are CD5 B cells. Both cases demonstrate diminished or absent response to typical foreign antigens (Hayakawa *et al.*, 1984b, Schultz and Greene, 1976; Sidman *et al.*, 1978). Admittedly, these response deficits may due to preselected pauciclonality, or to an altered differentiation stage in CD5 B not allowing priming (as compared with other virgin B cells). However, the observations with CD5 B cells from both strains, high autoantibody secretion and decreased response to foreign antigens, suggest that the CD5 B cells present in adult mice very likely play only a limited role in typical antibody responses to foreign antigens, but rather are enriched for autoantibody specificities.

Furthermore, the responses of these cells in normal mice have been tested by using immunoglobulin allotypes to mark cell populations wherein peritoneal cavity CD5 B cells reconstitute CD5 B cells in irradiated or newborn mice. Again, reconstituted CD5 B cells showed restricted responses. Responses to DNP or NP coupled to TI or TD carriers were decreased (Forster and Rajewsky, 1987; Lalor and Morahan, 1990). Lalor carried out a limiting-dilution assay using sorted peritoneal cavity CD5 B cells and reported enriched reactivity to several antigens, but only low-affinity reactivity to typical haptens (Lalor and Morahan, 1990), suggesting a difference in repertoire. However, these results could also be explained by a bias resulting from further selection during repopulation of recipients or during the generation of the surviving adult population. Therefore, the question of the initial repertoire of CD5 B cells and the capacity of these newly generated cells (versus the adult selected population) to participate in T-dependent and T-independent responses remains an area of active investigation.

VII. Biases in V Gene Usage

The biases in specificity described above can sometimes be identified by analysis at the V gene level. For example, as mentioned earlier, the anti-BrMRBC specificity is predominantly encoded by two V gene combinations, V_H11 in association with $V_\kappa9$ and V_H12 in association with $V_\kappa4$ (Reininger *et al.*, 1988; Hardy *et al.*, 1989; Mercolino *et al.*, 1989; Pennell *et al.*, 1989a). Analysis of immunoglobulin V gene utilization can provide strong evidence for the relatedness of normal and malignant CD5 B cells, particularly if the analysis is focused on V genes associated with such specificities enriched in normal CD5 B cells. It should be stressed that mouse CD5 B cells have been shown to

express a large set of V_H genes (from all known V_H families) as estimated by V_H family usage (Hayakawa *et al.*, 1988; Andrade *et al.*, 1989). Their repertoire, although perhaps much less diverse than that of $CD5^-IgD^{++}$ B cells, is thus still quite broad.

A. V Gene Biases in Normal CD5 B Cells

Jaton's group found repetitive usage of a distinctive V_H/V_L combination in anti-BrMRBC specific hybridomas made from peritoneal cavity cells of NZB and C3H mice (Reininger *et al.*, 1987). Furthermore, 14 of 16 anti-BrMRBC hybridomas made with CD5 B cells sorted from the spleens of SM/J mice also utilized this same combination (Hardy *et al.*, 1989). This V_H gene is a member of the small (two to three members) V_H11 gene family and is associated with a member of the $V_\kappa9$ family in these hybridomas. Two other hybridomas in this set utilized the V_H12 gene, a single-member family that Clarke had found was utilized frequently in peritoneal hybridomas with this specificity in $B10.H\text{-}2^aH\text{-}4^b$ mice (Pennell *et al.*, 1989a). Neither V_H11 nor V_H12 had been encountered previously in association with other specificities. Mapping studies revealed that neither family was among the more J_H-proximal V_H genes (Hardy *et al.*, 1989; Pennell *et al.*, 1989b). This latter observation was important since J_H-proximal V_H genes have been hypothesized to preferentially encode self- and/or environmental reactivities and also to be overrepresented in early B cells (Yancopoulos *et al.*, 1984; Perlmutter, 1987).

The enrichment of V_H11-encoded anti-BrMRBC specificity in CD5 B cells has been demonstrated with normal cells of BALB/c mice by PCR analysis of V_H11 to J_H1 or J_H4 DNA rearrangement (Carmack *et al.*, 1990). This type of analysis, utilizing DNA, avoids potential complications in experiments measuring RNA where striking differences in the levels of message can occur between resting and activated cells. V_H11/J_H1 rearrangement was shown to be enriched in CD5 B cells from both spleen and peritoneal cavity when compared to either bone marrow pre-B cells or $CD5^-IgD^{++}$ B cells from either spleen or peritoneal cavity. These data provide conclusive evidence demonstrating a difference in repertoire between distinct IgM^+ B cell subsets, even when located in the same tissue. They also showed that V_H11 is rearranged in bone marrow (and its progency, the $CD5^-IgD^{++}$ cells in spleen) at about the frequency that would be expected for a small V_H family. Importantly, they revealed that such V_H11 rearrangement seen in bone marrow and spleen $CD5^-$ B cells utilized all J_H segments, in contrast with extreme J_H1 preference in CD5 B cells (likely due to selection).

The same study also reported, curiously, that anti-BrMRBC antibody labeled that B cells capable of secreting anti-BrMRBC antibody (Carmack *et al.*, 1990). This fraction is also the population that binds PtC vesicles (Hayakawa *et al.*, 1992). This specific staining was interpreted as due to occupancy of the anti-BrMRBC Ig receptor by multivalent antigen, which then allowed specific "sandwich" staining. Thus one reason for preferential selection and maintenance of CD5 B cells is likely the availability of antigen. Certain autoantigens may serve as such antigens if corresponding autoantibody specificities are encoded by germ-line genes rearranged in CD5 B cells. In addition, the enriched level of V_H11–J_H1 rearrangement was restricted to this anti-BrMRBC$^+$/PtC binding fraction of CD5 B cells, again consistent with enrichment based on selection (Carmack *et al.*, 1990; Hayakawa *et al.*, 1992).

Thus one explanation for the overrepresentation of V_H11/anti-BrMRBCs in CD5 B cells appears unlikely, that the promoter/enhancer structure restricts its rearrangement to fetal B cells. Together with the finding that V_H11 (and V_H12) are not located proximal to D, it would appear that the repertoire of CD 5 B cells may be strongly influenced by antigenic selection during the neonatal period. More recently, Pennell and Clarke have reported an excess of S107 family genes, particularly V11, in a panel of hybridomas derived from peritoneal cells of the B10.H-2^aH-4^b strain mouse (Pennell *et al.*, 1990). Again, they attribute this increased gene expression to selection, particularly since V_L association was nonrandom.

V gene sequence analysis of a series of antithymocyte hybridomas derived from sorted CD5 B cells demonstrated a more diverse usage of combining sites (Hayakawa *et al.*, 1990)—14 ATA hybridomas derived from CD5 B cells utilized V_H genes belonging to four distinct families (J558, 3609, Q52, and Vgam3.8). Notably, fine specificities correlated with usage of particular V_H genes. Three groups of ATAs used nearly identical V_H genes, one utilizing 3609, another with a J558 member, and a third with a different J558 gene. Two hybridomas derived from a clonal expansion. However, repetitive usage of identical V_H genes not due to clonal proliferation was also found in ATAs. For example, the four V_H3609 hybridomas were unrelated, as shown by D and J_H differences. Another pair used identical V_H and J_H genes, but were unrelated as distinguished by their D genes. The observation of similar binding specificity with identical V_H gene usage by clonally unrelated cells suggests that ATA-specific B cells were enriched among CD5 B cells primarily due to their specificity, likely by an antigen-driven selection mechanism. The nature and origins of these antigens are not

clear, although many appear to include carbohydrate. Thus, as with the anti-BrMRBC/anti-PtC specificity, it seems likely that natural ligands (environmental antigens or autoantigens) are the most likely candidates for long-term constant selection of these specificities in CD5 B cells.

B. V Gene Usage in CD5 B Cell Clonal Expansions or Neoplasias

Remarkably recurrent V gene usage by CD5 B cells has been found in analyses of premalignant, reconstituting, and malignant cells. As opposed to normal CD5 B cells, surprisingly limited usage of V_H (and V_L) genes was observed in a collection of 10 mouse $CD5^+$ B lymphomas (CH lines: Haughton *et al.*, 1986), implying nonrandom expression (Pennell *et al.*, 1988). These V genes all appear unmutated and have served as prototypes for studies of selected V gene usage by normal CD5 B cells. Intriguingly, V_H11 is overrepresented in the CH cell lines (3/10) and has also been found in three independently established $CD5^+$ *in vitro* B cell lines that occurred spontaneously in long-term culture (Braun and King, 1989).

Furthermore, a V_H gene used in a CH lymphoma has also been described in CD5 B cells at a premalignant stage. Southern analysis of immunoglobulin gene rearrangement in the expanded B cell population from BWF_1 mice (Tarlinton *et al.*, 1988) in addition to studies of the tumorigenic potential of hyperdiploid (mostly $CD5^+$ B) cells in NZB mice (Raveche *et al.*, 1988) strongly suggest that the uniquely elevated $CD5^+$ B cell population found in BWF_1 or NZB mice is in a premalignant stage with oligoclonal proliferation. A V_H gene utilized predominantly in the CD5 B cells in a BWF_1 mouse (showing clonal expansion) has been sequenced. This V_H was found to be nearly identical to that seen previously in the $CD5^+$ B lymphoma CH12 (Tarlinton *et al.*, 1988). The work of Herzenberg and Stall has shown that clonal expansions of CD5 B cells in the peritoneal cavity are a frequent event in aged normal strains and occur much earlier in the BWF_1 background (Stall *et al.*, 1988). These expansions can yield lines that are easily adapted to culture. Thus, it appears clear that CD5 B cells have a propensity for generating cells with less regulated growth and even neoplasia (at least in some strains).

Repeated utilization of distinctive V genes has been documented in both normal CD5 B cells and in B lymphomas by analysis of B cells reestablished after peritoneal cell transfer into neonatal recipients. Forster *et al.* (1988) made hybridomas from recipients that had received normal peritoneal cells (enriched for CD5 B cells), and then examined V gene utilization. This experiment was carried out based on

previous data that CD5 B cells have the ability to self-reconstitute and likely select for those CD5 B cells with the greatest proliferative potential (Forster and Rajewsky, 1987). One recipient mouse developed a B lymphoma whose origin could be traced to the transferred peritoneal cells. The light chain of this immunoglobulin was identical to the $V_\kappa 9$ employed in the $V_H 11$ anti-BrMRBC specificity from CD5 B cells and to that also found in one of the previously studied B lymphomas, CH34. Two other sets of hybridomas were established from individual reconstituted mice. Out of 14 hybridomas analyzed, most (12/14) were identified as utilizing nearly identical V_H or V_L sequences (either together or singly) as had been seen earlier in the CH series of $CD5^+$ B lymphomas.

Although enrichment of particular V_H and/or V_L genes has been clearly documented in expanded $CD5^+$ B cell populations or neoplasias, the origin of this bias remains unclear. Is this preferential utilization simply due to the specificity generated by expression of particular V_H and/or V_L gene combinations or instead is it due to preferential rearrangement of such V genes? Certainly, antibody specificity is likely to play an important role because the usage of identical V_H/V_L combinations has been observed in several clonally independent CD5 B cells and tumors (Pennell *et al.*, 1988; Hardy *et al.*, 1989; Forster *et al.*, 1988). The occurrence of a population of BrMRBC-binding CD5 B cells in normal young adult mice utilizing two distinctive V_H/V_L combinations provides one of the clearest examples of such an enrichment. Whether the self-reactivity encoded by these distincitve combinations contributes to the expansion or neoplastic transformation or instead simply reflects a preexisting bias in a B cell subset susceptible to disregulated growth remains an unresolved issue.

Thus the rearrangements frequently seen in $CD5^+$ B cell neoplasias likely reflect the preexisting biased V gene usage found in the normal CD5 B cell population. Furthermore, it appears clear that antigenic selection plays an important role in establishing this enrichment. However, the mechanism whereby such enrichment would be confined to CD5 B cells, and whether preferential V gene rearrangement might play a role in this restriction (perhaps altering the initial repertoire) are unknown. Considering the fetal origins of most or all CD5 B cells, then a comparison of the rearrangement process in fetal and adult development may prove illuminating.

C. Distinctions in Rearrangements

The frequency of N-region addition in Ig sequences from B cells generated early in ontogeny is generally low when compared to sequences from the adult (Feeney, 1990). Thus, the degree of N-region

addition might serve as a hallmark for whether a lymphocyte is generated early or later in ontogeny. Sequence analysis of anti-BrMRBC and ATA hybridomas has shown decreased N-region addition, but there are numerous examples of rearrangements with some N addition. Rajewsky and colleagues also found a difference in N-region size between CD5 B cells from neonates and adults (Gu *et al.*, 1990), suggesting accumulation of some cells with N addition over time. They interpreted this to mean that some CD5 B cells are derived later in development and so have more N-region addition. However, alternatively, it is possible that for certain highly selected specificities, CD5 B cells lacking N-region addition could be at a disadvantage in establishing progeny in the adult. Then infrequent cells with some N-region would become overrepresented in the final adult repertoire. This issue will only be resolved by more extensive sequence analysis, particularly of populations of B cells generated from progenitors of known ontogenic origins (such as fetal and adult pro-B cell-reconstituted SCID.

Regardless of whether fetal B precursors have no or only diminished N-region addition, this feature suggests that the primary repertoire of such cells will differ from that of newly generated bone marrow B cells. Thus, some have suggested that in the absence of N addition short homologous sequences might play an important role in biasing V to D and D to J rearrangement (Gu *et al.*, 1990). Alternatively, short inverted repeats, known as "p elements," might generate biases, as has been shown in rearrangements of γδ T cells (Lafaille *et al.*, 1989). Furthermore, the possible contribution of preferential rearrangement of J-proximal V genes (Yancopoulos *et al.*, 1984) remains to be assessed. Clearly, the finding that selection is critical in shaping the adult CD5 B cell repertoire does not eleminate in the initial repertoire potentially important distinctions that might exist between fetal and adult B cells, and more work is necessary to decide this issue. More speculatively, re-rearrangement of V_H or V_L (from the original V_H–V_L combination) might occur, as has been seen with a $CD5^+$ B lymphoma (Hardy *et al.*, 1986; Kleinfield *et al.*, 1986). Certainly this should be a fertile field for investigation in the next few years, particularly as new technologies such as single-cell sequencing become widespread and routine.

D. CD5 B Cells in Immunoglobulin-Transgenic Mice

If selection is critical in determining the CD5 B cell antibody repertoire in adult mice, then this may provide an explanation for curious observations in immunoglobulin-transgenic mice regarding the degree of exclusion of endogenous rearrangement in different B cell

subsets. Analysis of endogenous expression in the M54 anti-NP μ-transgenic line revealed decreased numbers of bone marrow and splenic B cells and significant numbers of endogenous-expressing B cells in the peritoneal cavity (Herzenberg *et al.*, 1987). This was interpreted as reflecting different rules for differentiation of $CD5^+$ and conventional B cells such that transgene expression had less of an effect on CD5 B cells. However, an alternative (not necessarily mutually exclusive) explanation for the increased endogenous Ig-expressing cells in CD5 B cell subset is that the anti-NP μ heavy chain does not provide a good antibody for selection into the adult long-lived population. In this view, infrequent fetal B cells that also rearrange their endogenous heavy chain gene are much more likely candidates for such selection and thus come to constitute the bulk of the adult population.

The issue of endogenous expression in immunoglobulin transgenic lines has generated a great deal controversy. More recent analysis of the M54 line suggests that many of these mice contain very significant numbers of bone marrow-derived (non-$CD5^+$) B cells in their spleens (Iacomini and Imanishi-Kari, 1992). In addition, crosses with mice bearing the X-linked immunodeficiency (xid) support the notion that most of these splenic cells are not related to CD5 B cells since xid eliminates most CD5 B cells, but spares many transgenic B cells in the spleens of these mice (Rabin *et al.*, 1992). On the other hand, the existence of "double-expressing" (endogenous and transgene) B cells has recently been confirmed by molecular analysis of hybridomas derived from peritoneal cells of the M54 line (Lam *et al.*, 1993). Possibly, analysis of lines bearing transgenes encoding specificities predicted to be selected for in the CD5 B cell subset (by appropriate self-reactivity) may help to shed more light on this issue. Thus, in V_H11 transgenic lines in which $CD5^-$ B cells in the spleen exclude poorly, there is better exclusion of the $CD5^+$ subsets in both the spleen and peritoneal cavity, supporting the importance of specificity in determining the degree of double expression (R. Hardy and K. Hayakawa, in preparation).

Immunoglobulin-transgenic mice have also been used in generating murine models of B cell tolerance. Nemazee has expressed an antibody reacting with $H\text{-}2^k$ MHC class I in both an $H\text{-}2^k$ (where it is autoreactive) and an $H\text{-}2^d$ (where it is not) background (Nemazee and Burki, 1989). He found deletion of B cells bearing the self-reactive antibody on the $H\text{-}2^k$ background. Goodnow *et al.* (1988) reported functional inactivation rather than deletion in an antilysozyme transgenic line when crossed with a mouse that constitutively expressed

lysozyme as a neo-self antigen. Neither of these groups has addressed the issue of CD5 B cells in their systems. Honjo has reported on a transgenic line that expresses a pathogenic antierythrocyte specificity (from a hybridoma that originated in an NZB mouse) as a model for autoimmune hemolytic anemia (Okamoto *et al.*, 1992). This group found variable numbers of sick individuals along with variable numbers of B cells in the spleen and peritoneal cavity. They further suggested that self-reactive B cells could survive, proliferate, and be activated to secrete pathogenic autoantibody in the peritoneal cavity since it was free of erythrocytes (Murakami *et al.*, 1992). Cells in the peritoneal cavity would die by apoptosis if erythrocytes were injected, suggesting an accumulation of self-reactive specificities in $CD5^+$ B cells by escape of deletion due to their distinctive anatomical location.

Unfortunately, this interesting study with anti-RBCs did not directly address the issue of whether these B cells were indeed CD5 B cells. This is critical since $CD5^-IgD^{++}$ cells can also reside in the peritoneal cavity. Furthermore, it is unclear whether this V_H/V_L combination is germ-line encoded, so this model may not provide much help in understanding the normal selection, differentiation, and activation of CD5 B cells. In fact, antierytherocyte autoantibody activity was not detected in the CD5 B cell population in normal mice, so this specificity is not characteristic of CD5 B cells (Hayakawa *et al.*, 1992). In addition, we have observed decreased numbers of CD5 B cells in the peritoneal cavities of Ig-transgenic lines encoding the high-affinity anti-dsDNA, suggesting that such combining sites fail to select cells into the adult CD5 B cell pool (R. Hardy, and M. Weigert, and J. Erikson, unpublished observations). These data suggest that certain pathogenic autoantibody specificities are not selected into the adult CD5 B cell subset, in striking contrast with the anti-BrMRBC/PtC-binding specificity enriched in V_H11 (or V_H12; Arnold *et al.*, 1993) transgenic mouse lines (R. Hardy and K. Hayakawa, in preparation).

VIII. A Homologous Human Population?

At about the same time as the identification of normal CD5 (then called Ly-1) B cells in the mouse, normal CD5 (then called T1 or Leu-1) B cells in the human were detected (Caligaris-Cappio *et al.*, 1982). Cell transfer experiments used to investigate the origins of CD5 B cells in mice are not possible in humans and so the relationship of these cells to the bulk of $CD5^-$ B cells has remained an issue. This is further complicated by the observation that CD5 can be up-regulated on B-CLLs, normal B cells, and normal T cells by culture with phorbol

ester (Miller and Gralow, 1984). Based on this fact, some have concluded that CD5 expression on B cells simply reflects activation (Freedman *et al.*, 1989). However, it is clear that B cells activated using a more physiological system (anti-Ig plus T cell-derived factors) do not necessarily induce detectable levels of CD5 in human (Hardy *et al.*, 1988). Several features of human CD5 B cells are also inconsistent with a cell activation stage explanation: (1) CD5 B cells predominate prior to birth before exposure to external antigen and decrease after birth when exposure may commence (Antin *et al.*, 1986); (2) CD5 B cell frequencies are quite stable for an individual and are not seen to vary with common infections (Kipps and Vaughan, 1987); and (3) CD5 B cells in peripheral blood do not show a surface phenotype typical of activated B cells (Bhat *et al.*, 1992). In the brief overview that follows, we touch on some similarities between mouse and human CD5 B cells and their association with certain diseases thought to have an autoimmune component. We end with an issue current in both mouse and human subsets, that of the V gene repertoire in normal and malignant CD5 B cells.

A. CD5 B Cells in Development and Disease

In the human, Bofill *et al.* (1985) reported (by tissue immunofluorescence analysis) that although CD5 expression was not found on very early surface IgM^-B-lineage cells or on IgD^-IgM^+ cells, CD5 B cells later constituted a large fraction of early lymph nodes after 17 weeks of gestation, and, furthermore, that some splenic B cells were $CD5^+$. Antin and Ault also found that CD5 B cells were not significant in fetal (~20 weeks) liver whereas they constituted a majority of B cells in fetal spleen (in contrast to their rarity in adult spleen) (Antin *et al.*, 1986). Furthermore, FACS analysis of mononuclear cells in cord blood (from 20 weeks and later) showed that a majority (50–95%) of B cells are $CD5^+$, whereas only 10–20% of B cells in adult peripheral blood lymphocytes (PBLs) are $CD5^+$ (Hardy *et al.*, 1987a). Bhat and Herzenberg reported that this relatively high frequency of CD5 B cells continues into childhood (50% or greater in children under 16), decreasing to the levels typical of adults only after late adolescence (Bhat *et al.*, 1992). Such CD5 B cells in early development rarely express CALLA antigen (Antin *et al.*, 1986). CALLA is detected on a larger proportion of fetal hematopoietic organs, immature lymphoid cells (Hokland *et al.*, 1983), and common acute leukemic cells (Burkitt's lymphomas) but not on B CLLs (Ritz *et al.*, 1981). Such mutually exclusive CALLA/CD5 expression carries significant implications, suggesting different etiologies of B cell malignancy depending on the original B cell subset affected.

There is intriguing data suggesting that the levels of circulating CD5 B cells in humans are under genetic control similar to the variation seen with different mouse strains. Kipps and Vaughan (1987) analyzed the frequency of CD5 B cells in a number of individuals, including monozygotic twins and triplets. They found variation between unrelated individuals, but relatively consistent levels for a given individual. Furthermore, very similar levels were seen in twins and triplets, even in cases wherein individuals were discordant for rheumatoid arthritis (where elevated levels of CD5 B cells have often been observed).

Genetic control of the frequency of human CD5 B cells is also suggested by association with certain diseases. There is a high incidence of individuals with elevated levels of CD5 B cells among patients with rheumatoid arthritis (Plater-Zyberk *et al.*, 1985; Hardy *et al.*, 1987a) or Sjögren's syndrome (Hardy *et al.*, 1987a; Dauphinee *et al.*, 1988). Sjögren's patients are known to have an increased risk of B cell malignancy. Furthermore, Fox *et al.* (1986) have suggested a transition of B cells from proliferation into neoplastic transformation marked by a cross-reactive idiotype (CRI) present on V_κIIIb of IgM rheumatoid factor (RF). Such Id^+ RF cells were found in a rheumatoid arthritis patient and shared with the RF the paraproteins in Waldenstrom's macroglobulinemia and lymphoma patients. By using the antibody to this CRI, they found progressive increases in the proportion of B cells bearing the CRI in patients with Sjögren's syndrome. Although not directly tested in this study, such proliferating B cells in Sjogren's patients may be the expanded population of CD5 B cells mentioned above.

B. CD5 B Cells: Autoreactivity, V Gene Usage, and Mutation

An enriched frequency of autoantibody associated with CD5 B cells was also evident in a study of human PBLs. Although CD5 B cells in human PBLs do not spontaneously secrete significant levels of immunoglobulin *in vitro* (Hardy *et al.*, 1987a), stimulation with *Staphylococcus aureus* induces IgM secretion. Comparing the IgM from cell fractions in which CD5 B cells are present or absent (purified by FACS) shows that one autoantibody (rheumatoid factor; IgM anti-IgG) is enriched 4- to 20-fold in the fraction containing CD5 B cells when compared with IgM derived from $CD5^-$ B cells (Hardy *et al.*, 1987a). This high frequency of RF specificities in CD5 B cells was also demonstrated using B cell lines transformed with Epstein–Barr virus (EBV) (from $CD5^+$ or $CD5^-$ B cells) (Casali *et al.*, 1987). The suggestion of RF secretion and CD5 B cells was foreshadowed by analyses showing that

increased levels of these cells are found particularly among patients with RA or Sjögren's syndrome, diseases in which RF secretion is often observed (Plater-Zyberk *et al.*, 1985; Hardy *et al.*, 1987a). Although levels of serum RF and CD5 B cells do not correlate (as for autoantibody with murine CD5 B cells in older NZB mice), if RF secretion comes preferentially from such cells, then individuals with genetic backgrounds leading to B cell activation may be especially prone to autoimmune disease if they also have (genetically regulated) elevated levels of CD5 B cells.

This repeated usage of particular V_H and/or V_L genes has also been found in human CLLs ($CD5^+$) and normal human CD5 B cells. It has been known for some time that autoantibodies (particularly rheumatoid factor) in different individual sera often share certain idiotypes (Capra and Keyhoe, 1974). By using antibodies to such idiotypes present either on V_L (17.109CRI on V_κIIIb) or V_H (G6CRI on V_H1), these idiotypes were found at high frequencies (~20% each, with half of the Id^+ cells expressing both) in B CLL but not in other types of $CD5^-$ B cell malignancies (Kipps *et al.*, 1988, 1989). Furthermore, normal CD5 B cells in peripheral tissues were also shown to contain a higher frequency of $17.109CRI^+$ or $G6CRI^+$ cells as compared to $CD5^-$ B cells (Kipps *et al.*, 1989), suggesting that the bias seen in CLL originates in the normal population (and providing further evidence connecting normal CD5 B cells with CLL). Further, these genes are enriched in fetal spleen cells (Kipps *et al.*, 1990). A comparison of EBV lines established from cord blood (a rich source of CD5 B cells) revealed high-level expression of the B_HIV gene family, but with different V_HIV-associated idiotypes associated with $CD5^+$ and $CD5^-$-derived lines (Mageed *et al.*, 1991). Curiously, many of these lines react with common self or environmental antigens, suggesting a similarity to murine CD5 B cells. Logtenberg and colleagues reported similar results with a different panel of EBV lines established from a 19-week-old fetus (Schutte *et al.*, 1991).

The finding of restricted Ig utilization or overexpression of particular V genes in fetal cells or in CD5 B cell-derived lines may be due to a biased initial repertoire that favors such reactivity or to selection for the reactivities that these genes encode. Biased rearrangement based on J proximity, thought to skew the repertoire of pre-B cells, appears not to be the explanation for these findings, as several of these genes belong to families that have been mapped to regions distal to J (Schutte *et al.*, 1992). Thus it appears likely that selection may operate very early (even by the second trimester of fetal development) to bias the initial J-proximal repertoire. This issue may be resolved eventually by

investigating whether biased V_H usage is accompanied by biased V_κ usage, that is, determining whether particular V_H/V_L combinations predominate.

Another issue that is being addressed in subsets of human B cells is the degree of somatic mutation that their V genes have accumulated. Most CLL genes are thought to have little or no somatic mutation and thus to express germ-line-encoded antibodies (Kipps *et al.*, 1988). The observation that many of these antibodies are directed to self or environmental antigens would then imply that even antigen binding does not result in accumulation of mutation. A possible exception to this observation is the CLLs expressing the V_HV family gene are often mutated, and in a fashion that suggests selection based on replacement/silent mutation ratios (Cai *et al.*, 1992). This same group reported that one of these V_HV genes showed much less mutation in cord blood B cells, adult peripheral blood B cells, and EBV-transformed $CD5^+$ B cell lines. Curiously, another group has reported little mutation in CLLs with V_HV usage (Rassenti and Kipps, 1993). Interestingly, analysis of V genes from small lymphocytic lymphomas (SLLs) that are $CD5^+$ and $CD5^-$ suggests that CD5 expression is found in those that express unmutated germline genes, whereas lines lacking CD5 often show intraclonal diversity and extensive somatic mutation (Pratt *et al.*, 1991). Thus, although there is considerable evidence that normal CD5 B cells and most B CLLs express germ-line antibodies, many of which react with self antigens such as RF or I, the explanation for the exceptional V_HV data remains as a major issue.

IX. Final Considerations and Summary

The induction of CD5 on B cells previously characterized as $CD5^-IgD^{++}$ by treatment with certain anti-IgM antibodies *in vitro* has called into question the significance of CD5 expression on B cells (Cong *et al.*, 1991). In the same study it was also shown that addition of IL-6 to the cultures more closely mimicked the phenotype of CD5 B cells found *in vivo*. These data led to the proposal of a "specificity" model for the generation of CD5 B cells, in which it is postulated that any B cell activated in a particular fashion (perhaps by TI–II type antigens) will give rise to $CD5^+$ progeny and that the CD5 B cells normally found *in vivo* are the result of such activation by environmental antigens. They further suggest that any difference in generation of CD5 B cells in the same environment but by different B progenitor sources (fetal versus adult) can be accounted for by differences in V gene usage and/or N-region addition that might distinguish these precursors.

As with any simple explanation (not invoking the existence of distinct B cell differentiation pathways), this model has its appeal. However, before deciding in favor of this model, several major assumptions need to be tested by experiment and some inconsistencies need to be accounted for. Thus, there is at present no evidence suggesting that a lack of N-region diversity results in a strong bias in reactivity toward self or environmental antigens. In fact, some of the V genes sequenced from murine CD5 B cells contain N additions (Hayakawa *et al.*, 1990). In addition, human Ig rearrangements contain extensive N additions at the time when CD5 B cells still predominate. The suggestion that TI–II antigens are critical is based on the observation that mice with the X-linked immunodeficiency (failure to respond to this class of antigens) also lack most functional CD5 B cells. However, it is clear that many B cells other than those expressing CD5 are affected by xid (Mosier, 1985). Finally, there is as yet no demonstration that immunization of mice with a TI–II type of antigen induces CD5 on the responding cells. In fact, very early data showed that the Ig-secreting cells induced by immunization with TNP–Ficoll, a classic TI–II type of antigen, are $CD5^-$ (Hayakawa *et al.*, 1984b) at least 3 days after immunization. Transient expression of CD5 by activation into cell cycle might explain this result; however, the majority of CD5 B cells are normally not proliferating (Forster and Rajewsky, 1987). Certainly many experiments remain to be done to allow incorporation of this CD5 induction result into the framework of an understanding of the generation of CD5 B cells.

In summary, we have suggested a model for $CD5^+$ B cell generation that regards fetal and adult B cell differentiation as significantly different, as shown in Fig. 1. This is already clear from differences in the expression of TdT and the recently described precursor lymphocyte myosin-like light chain (Oltz *et al.*, 1992) during the pro-B cell stage of differentiation (Li *et al.*, 1993; Oltz *et al.*, 1992). We propose (Fig. 2) that the B cells generated from fetal precursors, besides expressing a somewhat different primary repertoire due to absence of N regions, also respond differently to cross-linking of their Ig: such cross-linking by germ-line encoded reactivity to self or environmental antigens results in selection into a long-lived mature pool. Since CD5 expression appears confined to IgM^+ cells *in vivo* or in culture, we also suggest that its expression may normally occur after the IgM^+ stage, perhaps even as a result of such positive selection. The difference of CD5 levels among fetal B cells, detectably $CD5^+$ or $CD5^-$ (which appear to be stable phenotypes), could reflect a differential threshold of such self antigen cross-linking in inducing CD5. In contrast, B cells generated from adult precursors respond to such cross-linking either

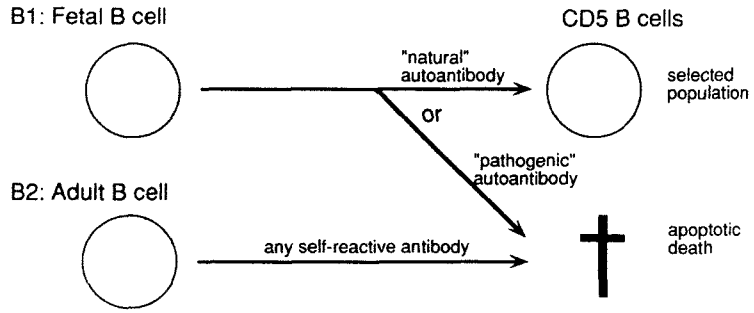

FIG. 2. Model for difference in B cell tolerance between fetal and adult newly generated B cell populations. In this view, fetal B cells are selected into the adult long-lived CD5 B cell population based on cross-linking of germ-line-encoded immunoglobulins. In contrast, similar cross-linking of newly generated bone marrow B cells results either in functional inactivation or in elimination.

by becoming anergized or by dying apoptotically. However, if a mutated high-affinity autoantibody specificity is introduced experimentally, a negative selection mechanism appears to operate in both fetal and adult B cells, as is suggested in transgenic mouse lines.

This model predicts that the signaling pathways operating in newly generated fetal and adult B cells will differ. Fetal B cells may posses a unique pathway to permit positive selection, or alternatively, may lack molecule(s) critically important in a negative selection mechanism. We are just beginning to dissect these pathways at the molecular level, but an obvious starting point for investigating developmental differences will be to study expression and function of the Ig-associated molecules (Hombach *et al.*, 1988) and of the tyrosine kinases (Dymecki *et al.*, 1990; Burkhardt *et al.*, 1991). It is possible that altered or even different molecules may be associated with surface Ig on certain B cell subsets, as some studies have suggested (Chen *et al.*, 1990; Yellen-Shaw and Monroe, 1992). Alternatively, characterization of neonatal B cell unresponsiveness to activation signals to which mature B cells respond may be a starting point in this investigation (Chang *et al.*, 1991; Yellen *et al.*, 1991). Furthermore, investigation of selection in the immature B cell compartment of the bone marrow, particularly in transgenic models of autoreactivity, may help to simplify this work (Hartley *et al.*, 1993).

This developmental view of B lymphopoiesis appears to have a counterpart in the T cell lineage in that the fetal thymus produces distinctive "waves" of T cells biased for particular receptors (with $\gamma\delta$ generally preceding $\alpha\beta$). In fact, it has been proposed that this differ-

ence of T cells constitutes a "developmental switch" (Ikuta *et al.*, 1990) similar to the familiar switch from fetal to adult hemoglobin in erythropoiesis (Papayannopoulou *et al.*, 1986; Whitelaw *et al.*, 1990; Wong *et al.*, 1986). We suggest that a comparable developmental switch is also occurring in B lymphopoiesis (Hardy and Hawakawa, 1991). Whereas the switch from fetal to adult hemoglobin is well characterized and understood, the biological significance of these developmental differences in the lymphoid system remains to be determined. Nevertheless, it is interesting to speculate that the immune system is gradually constructed during ontogenic development (perhaps reflecting phylogenetic progression?), primarily directed toward self/environmental antigens neonatally, but eventually generating a system efficient for defense against foreign antigens.

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Human Natural Killer Cells: Origin, Clonality, Specificity, and Receptors

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I. Introduction

Natural killer (NK) cells represent a third population of lymphoid cells (1, 2). They substantially differ from T or B lymphocytes in that they do not express (or rearrange) known receptors for antigen, namely surface immunoglobulin (Ig) and the CD3/TCR complex (2). They are phenotypically characterized by the surface expression of low-affinity receptors of IgG (FcγRIII or CD16) (3) and by the NCAM-homologous CD56 molecules (4–8). In contrast to resting T or B lymphocytes, NK cells are relatively large and have typical cytoplasmic azurophilic granules. Because of these morphological features they have been termed "large granular lymphocytes" (LGLs) (9). NK cell maturation can occur in the absence of a functional thymus (10). Indeed, functional NK cells are present in nude mice as well as in severe combined immunodeficiency (SCID) mice (11, 12). Whereas NK cells are spontaneously able to mediate their effector function (i.e., with no need for prior activation *in vitro* or *in vivo*), resting T or B lymphocytes cannot. It is for this reason that natural killer cells have been so named (13).

In general, NK cells have been considered as effector cells that lyse tumor cells or virally infected cells via nonspecific mechanisms (1). Importantly, both their cytolytic effect and the spectrum of their antitumor activity are greatly increased after culture of NK cells in interleukin-2 (IL-2). Indeed, IL-2-activated NK cells represent the most potent effector cells among the so-called lymphokine-activated killer (LAK) cells (1, 14–17). Both the NK and LAK cell-mediated functions have been defined as being non-MHC restricted (18). This definition is because of their ability to lyse efficiently those tumor cells that lack surface expression of MHC molecules, such as the K562 erythroleukemic cell line, or those expressing unrelated MHC class I molecules (1, 19). The apparent lack of specific receptors for antigen, together with the observation that the various NK functions (e.g.,

cytolytic activity, lymphokine production) are mediated by essentially all NK cells, have been considered as strong arguments in favor of the lack of clonally distributed functions in NK cells.

However, several of the generally accepted theories concerning NK cells have been challenged by a evidence based on a variety of new experimental approaches and technological breakthroughs. In this context, in humans, important progress has been possible since cloning of NK cells has become available (20, 21), and this has been applied to the study both of functional capabilities and of surface molecules with receptor function. Thus, based on the clonal approach, a number of surprising results have been obtained that have substantially changed our concepts of NK cells. This review is intended to summarize and discuss these findings. Namely, we review data showing that NK cells have the following characteristics.

1. NK cells may originate from precursors present in early fetal liver (6–8 weeks) and even in immature postnatal thymocytes.
2. They display specific functions (i.e., specific lysis of certain allogeneic cells).
3. They lyse not only tumor cells but also some normal allogeneic cells.
4. They display clonally distributed functions (i.e., recognize different allospecificities). Perhaps more importantly, several NK clones, displaying different allospecificities, can be derived from single individuals, thus providing evidence for the existence of an NK cell repertoire.
5. They specifically recognize class I molecules. Different from T cells, binding of NK cells to class I molecules appears to result in a "negative signal" leading to target cell protection.
6. They express clonally distributed functional receptors mediating specific recognition of class I alleles.

II. Ontogeny of NK Cells

It is well established that commitment to the B or T cell lineage is characterized by irreversible rearrangement of the immunoglobulin or TCR genes, respectively. In contrast NK cells do not rearrange Ig or TCR genes (22–27) and can undergo phenotypic and functional maturation in SCID mice (12, 28, 29). In addition, they undergo normal development in the absence of a functional thymus (11). Despite the "thymus independence" and the lack of a TCR rearrangement, NK cells share with T cells a number of properties regarding the expres-

sion of surface markers and effector functions, including cytolytic activity and lymphokine production (1). These data have supported the notion that T and NK cells may belong to related lineages.

A number of recent data may help to better understand the relationship existing between the two cell lineages. These data have been obtained by the analysis of lymphocyte differentiation during fetal ontogeny, as well as by the development of *in vitro* systems that mimic the *in vivo* environments necessary for NK or T cell maturation from precursors that are present either in human postnatal thymus or in fetal liver (30–32).

Rodewald *et al.* have described a murine population of early fetal thymocytes with the potential to differentiate toward T or NK cell lineages, depending on the *in vivo* microenvironment (33). Thus, a predominant thymic population has been detected at 14.5 days of gestation; these cells lack both CD4 and CD8 differentiation antigens, but express FcγRII/III several days before the expression of TCR. When this population was maintained in a thymic microenvironment it underwent differentiation toward mature T cells. On the other hand, when removed from the thymus this population selectively generated functional NK cells *in vivo* as well as *in vitro*. These data clearly indicate that a typical marker of mature NK cells is expressed early during T cell ontogeny. More importantly, they indicate that an immature thymocyte population gives rise to T or NK cells depending on the microenvironment (Fig. 1).

In agreement with these data, we showed that human immature thymocytes (isolated during cardiac surgery from patients ranging in age from 2 months to 4 years) characterized by the $CD7^+3^-4^-8^-$ phenotype could undergo *in vitro* maturation toward T or NK cell lineages (34, 34a). Again, the pattern of maturation was strictly dependent on the type of microenvironment and stimuli provided. Thus, growth of $CD3^-16^+$ cells with strong non-MHC-restricted cytolytic activity was obtained both at the population level and at the clonal level in the presence of exogenous IL-2 and the H9 leukemia cell line as a source of irradiated feeder cells (Fig. 1). Since over 50% of clones obtained under limiting dilution conditions were phenotypically and functionally undistinguishable from typical NK cell clones derived from peripheral blood, these data clearly indicated that precursors of NK cells are present within the human thymus. A possible explanation for the H9 cell dependence may be the involvement of cytokines and/or cell surface molecules, expressed by H9 cells, that become active on NK cell maturation/proliferation. Thus, H9 cells would mimic the microenvironment required for the physiological NK cell development. In

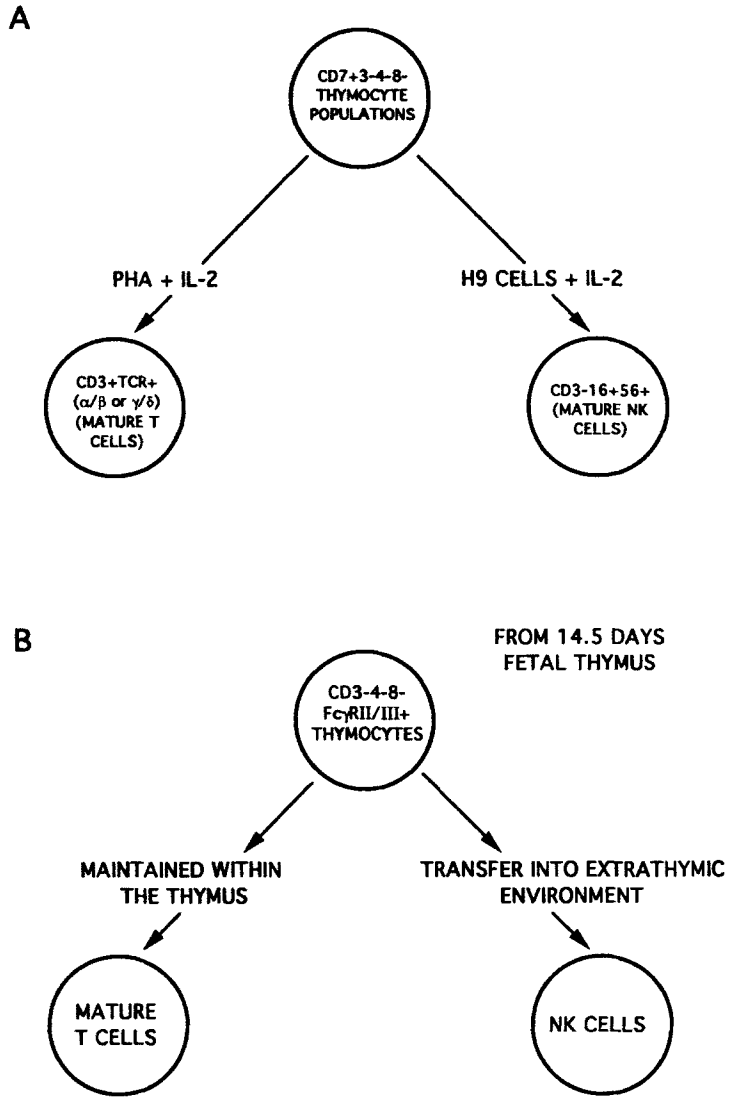

FIG. 1. Thymocytes contain precursors of both T and NK cells and their development toward T or NK cells depends on the *in vitro* or *in vivo* microenvironment. (A) Human $CD7^{+}3^{-}4^{-}8^{-}$ thymocyte populations isolated from postnatal thymus can undergo *in vitro* maturation toward mature T (either TCR $\alpha\beta$ or $\gamma\delta$) (34a, 35a) or NK lymphocytes (34), depending on the stimuli and the feeder cells. These experiments have been performed both at the population level and at the clonal level. (B) *In vivo* experiments in the mouse. In this case, the thymic populations were isolated from day 14.5 fetal thymuses. These populations were also characterized by the expression of FcγRII/III. When these cells were maintained within a thymic microenvironment, they developed into mature T cells. In contrast, when transferred into an extrathymic environment, they underwent maturation toward NK cells (35). Taken together, these data suggest that the precursors of both T and NK cells can derive from a phenotypically homogeneous subset of thymocytes.

this context, proliferation of $CD16^+$ cells from immature thymocytes has been obtained in the presence of thymic stromal cells as a source of feeder cells (35). The same $CD3^-4^-8^-$ populations giving rise to mature NK cells, when cultured in the presence of phytohemagglutinin (PHA) and IL-2, gave rise to $CD3^+TCR^+$ mature T cell populations that expressed $\gamma\delta$ functional receptors (34a). Development of TCR α/β^+ cells has been reported by another lab (35a).

A similar approach was applied to the study of lymphoid precursors present in the human liver isolated from embryos or fetuses at 6–10 weeks of gestation (36). In the presence of H9 cells and IL-2, liver cell suspensions underwent both proliferation and maturation toward $CD3^-16^+$ NK cells (undistinguishable from polyclonal populations or clones derived from adult peripheral blood). Importantly, the precursors of NK cells were restricted to a small population expressing the common leukocyte antigen CD45 (2–20% of all cells). These data indicate that precursors capable of *in vitro* differentiation toward NK cells can be identified as early as 6 weeks of gestation. Unexpectedly, by applying to liver cell suspensions the "*in vitro* microenvironment" able to induce T cell maturation from thymic precursors, we could obtain cell proliferation and maturation toward $CD3^+TCR\alpha\beta^+$ or $CD3^+TCR\gamma\delta^+$ cell populations or clones. It should be noted that these data could be obtained also in embryos at 6–8 weeks of gestation—that is, before colonization of the thymic rudiment by T cell precursors. The gestational age together with the finding that $CD3^+TCR^+$ cells could not be generated from cells isolated from the upper part of thorax of the same embryos clearly indicate that liver-derived proliferating lymphocytes are not thymic emigrants, but rather they are derived from liver precursors. Again, the T cell precursors were confined to the small $CD45^+$ subset of liver cells. It should be emphasized that these data provide the first experimental evidence that mature T cells can derive *in vitro* from early fetal liver precursors without a functional thymus and may provide insights in the mechanism of T cell maturation and selection. Interestingly, similar data have been reported by Sanchez *et al.*, who used different culture systems (37).

Taken together, data on mouse and human thymocytes, as well as human fetal liver, indicate that these cell populations may contain precursors that are committed either to the T or to the NK cell lineage: if this is the case, appropriate maturation signals would be sufficient for these committed precursors to undergo maturation to T or NK cells. An additional explanation, which is equally compatible with available data, is that the thymus and/or fetal liver contain uncommitted lymphoid precursor or pluripotent stem cells. This would imply that the

culture conditions used *in vitro* would mimic the microenvironment (e.g., thymus) needed to induce differentiation of uncommitted precursors toward T or NK cells. Data from studies of mice and humans do not clarify whether T or NK cells are derived from the same or different cell precursors. It is clear that the solution to this problem would be possible only after the development of culture conditions that allow, in single-cell assays, optimal maturation toward different hematopoietic cell lineages, including T and NK cells.

III. Expression of CD3 Genes during NK Cell Maturation

Although a major discrimination between mature T and NK cells is the rearrangement and the expression of a functional TCR/CD3 complex, nevertheless certain molecules of the CD3 complex are also expressed in NK cells (38). Thus, CD3 ζ chains have been shown to be associated with CD16 (39–41), CD2 (42), or p58 (43) molecules (see later). In addition, transcripts for CD3 ε have been described in both polyclonal and clonal NK cell populations (44). Using antisera raised against a CD3 ε deduced peptide sequence, Lanier and co-workers detected the CD3 ε protein at the cytoplasmic level (45, 46). In no instance could transcripts for CD3 γ and δ be detected in resting or activated mature NK cells.

Analysis of the expression of the various CD3 chains at the level of embryonic/fetal NK cells showed that, in addition to the ζ chain, CD3 ε and CD3 δ chains and a small amount of CD3 γ chain (46) could also be detected at the cytoplasmic level. Moreover, analysis of large numbers of $CD3^-16^+56^+$ NK clones derived from immature thymic populations ($CD3^-4^-8^-$), under appropriate culture conditions (see above), revealed, in a portion of NK clones, cytoplasmic CD3 ε proteins as detected with conventional anti-CD3 monoclonal antibody (mAb) (specific for the CD3 ε chain) (34). Thus, it appears that the precursors of NK cells express a set of CD3 proteins that is more closely related to mature T cells than to mature NK cells (34, 45). These findings suggest, but do not prove, that the precursors for the two cell lineages either share a common set of activated genes or are identical. In the latter case (i.e., if T and NK cells arise from a common precursor) the TCR rearrangement in T cells would be accompanied by the expression of the full set of CD3 genes, whereas maturation toward mature NK cells would be characterized by the down-regulation of CD3 γ and δ genes (38).

IV. Evidence for NK Cell Specificity

The first demonstration that human NK cells recognize normal allogeneic cells in a specific fashion was obtained in experiments in which T cell-depleted ($CD3^-$) peripheral blood lymphocytes were stimulated in a mixed lymphocyte culture (MLC) against irradiated allogeneic peripheral blood lymphocytes (47). Recombinant IL-2 was added as a source of exogenous growth factor. The resulting NK lymphoblasts were characterized by the $CD3^-16^+56^+$ surface phenotype and displayed a strong cytolytic activity against a series of NK-susceptible as well as NK-resistant tumor target cells. Surprisingly, these cells also lysed PHA-induced blasts derived from the allogeneic donor used as the stimulator in a MLC. On the other hand, lysis was not detected against autologous or against some unrelated allogeneic PHA blasts. Further clonal analysis clearly demonstrated that the ability to lyse specifically a given type of allogeneic target cell is a clonally distributed function. Thus, only a fraction of MLC-derived NK clones displayed the ability to kill the stimulating allogeneic cells (48). On the other hand, all MLC-derived NK clones had a similar ability to kill K562 target cells (47). Interestingly, NK cells that specifically killed normal allogeneic endothelial cells have been described by Bender *et al.* (49, 50). Also in this case, NK cells were cultured in the presence of allogeneic (endothelial) cells and IL-2, and then tested for specific cytolysis.

V. The Human NK Cell Repertoire

MLC-derived NK clones obtained from different donors displayed different patterns of cytolytic activity against a series of normal allogeneic target cells (48, 51). These data suggested the existence of different NK specificities in different individuals. A more fundamental question was whether clones with different specificities could be obtained from the NK cells of single individuals. In these experiments, NK clones were derived directly from peripheral blood $CD3^-$ lymphocytes. Clones were analyzed for cytolytic activity against a panel of normal target cells (PHA blasts) derived from different allogeneic donors. In the representative donor A, 36% of the 415 NK clones analyzed displayed cytolytic activity against one or more of the allogeneic target cells of the panel. In Table I, the cytolytic patterns of five distinct groups of alloreactive NK clones, four of which derived from donor A, are shown. It is evident that each group of clones displays a unique

TABLE I
EVIDENCE FOR AN NK CELL REPERTOIRE IN HUMANS

Alloreactive NK clone group[a]	Normal donor target cells (PHA blasts) lysed[b]						
	A	1	2	3	4	5	6
1	+	−	−	−	−	−	+
2	−	+	+	+	−	+	−
3	−	+	+	−	+	−	+
4	−	+	+	+	+	−	−
5	−	−	−	−	−	−	+

[a] The various groups of NK clones were defined on the basis of their patterns of cytolysis against a panel of normal allogeneic PHA-induced blast cells. It is of note that group 1 clones were derived from donor 1, whereas clones from groups 2–5 were derived from donor A.

[b] +, susceptibility to lysis; −, resistance to lysis.

pattern of cytolytic activity against the allogeneic donors. It is also noteworthy that none of the clones lysed autologous PHA blasts. These experiments provided evidence for the existance of a repertoire for alloantigen recognition in NK cells (51). It should also be noted that experiments reported by Bender *et al.* on the lysis of allogeneic endothelial cells could be interpreted on the basis of the existence of (at least) two distinct NK specificities in a single donor (50). In our studies, four NK-defined specificities have been determined, in a single donor; however, because more than 60% of NK clones isolated from this donor did not lyse any of the target cells of the panel, it is possible that a wider NK cell repertoire exists. Perhaps the use of a larger panel of allogeneic target cells might allow further definition of groups of alloreactive clones. It is also possible that a major subset of NK cells lacks the capability to lyse normal allogeneic cells (for possible interpretations, see Section VIII, regarding clones with broad specificity for MHC class I molecules). In any event, the actual amplitude of the human NK cell repertoire remains an open question.

VI. Genetic Analysis of Human NK-Defined Allospecificities

Given these results, the next major goal was identification of the molecules expressed on normal target cells that are involved in the mechanism of susceptibility or resistance to NK cell-mediated lysis. In fact, by the use of alloreactive NK clones, it has been possible to distinguish (for each group of clones) two different phenotypes, i.e., "susceptibility" and "resistance" to lysis (48). Genetic analysis has been performed in several informative families (51). PHA blasts were

derived from the various family members and assessed for their susceptibility to lysis by NK clones belonging to the various specificities. By this analysis it has been possible to define the mode of inheritance of the characters "susceptibility" or "resistance" to lysis by different alloreactive clones. These studies allowed the following definitions:

1. The various characters, "susceptibility to lysis" by different groups of NK clones, segregate independently and are inherited in an autosomal recessive mode. In contrast, the opposite characters, "resistance to lysis," are inherited in a dominant fashion. These data support the existence of specificities recognized by each group of NK cell clones.
2. The various NK-defined characters cosegregate with certain HLA haplotypes (or, more precisely, there is not an independent sampling between the two genetic traits). This implies that the genes that govern susceptibility or resistance to lysis are on chromosome 6.
3. Some donors are susceptible to more than one group of alloreactive NK cell clones, indicating that single individuals express an NK-defined complex haplotype. For example, in Table I, donors 1 and 2 are susceptible to lysis by three groups of NK clones.

VII. Involvement of Class I Molecules in NK-Specific Functions

Several experimental results provide evidence suggesting that class I molecules expressed on target cells may play a role in NK-mediated cytolysis. Thus, an inverse correlation has been described between expression of class I antigens on target cells and susceptibility to NK cell-mediated lysis. For example, target cells such as the class I-negative YAC-1 (in mice) and K562 (in humans) are highly susceptible to NK-mediated lysis (52–54). Variants of YAC-1 cells with class I expression became resistant to NK cells (52). A similar effect was observed in K562 cells on induction of class I expression by interferon γ (IFN-γ) (55). On the other hand, these concepts were challenged by the observation that tumor cells expressing HLA class I antigens were susceptible to lysis by NK cells and/or by $CD3^-16^+$ LAK cells. Kärre *et al.*, (56, 57) suggested that in autologous combinations, NK cells kill target cells because they express reduced levels of, or lack, self MHC class I molecules (58). These data, together with our finding that the NK-defined characters, susceptibility/resistance to lysis, cosegregate with HLA haplotypes and thus are carried on chromosome 6, focused our attention on the MHC region. Therefore, we analyzed informative families in which one of the donors had a recombinant MHC haplo-

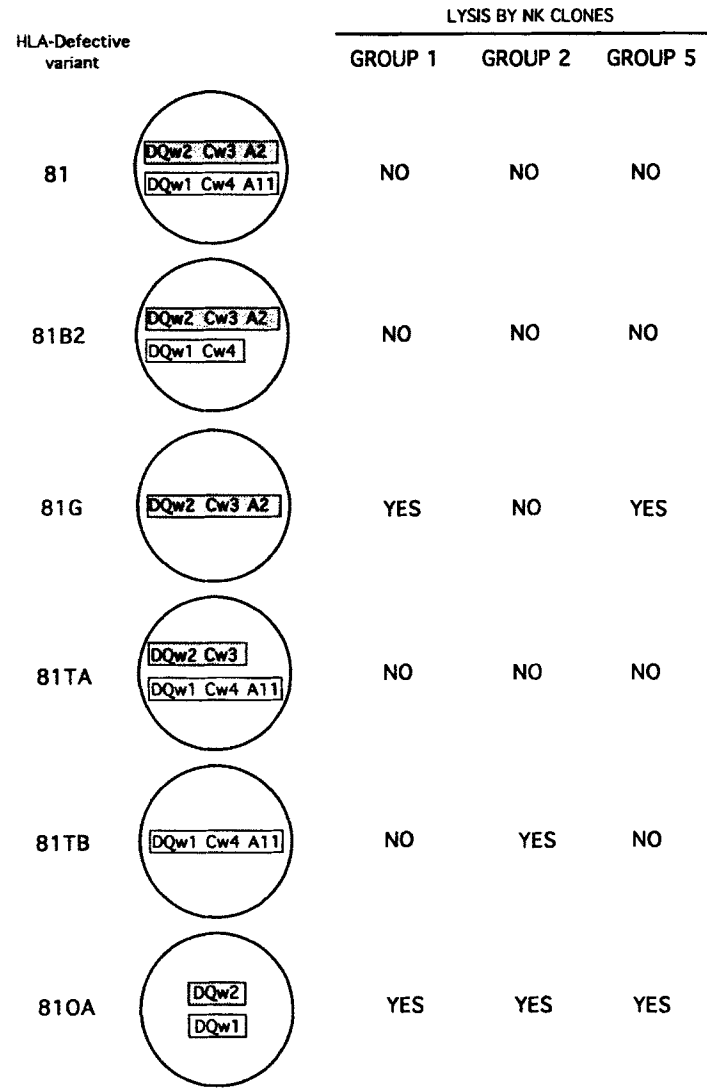

FIG. 2. Susceptibility to lysis by alloreactive NK clones of variants lacking one or more HLA allele. The parental EBV cell line 81 was derived from a donor heterozygous, and thus resistant to lysis, for three distinct NK-defined specificities (1, 2, and 5). A schematic representation of the HLA alleles expressed by each variant is shown. The paternal (bottom lines, and 81TB) or maternal (top lines, and 81G) chromosome 6 is schematically indicated for each variant. HLA-defective variants were analyzed for susceptibility (YES) or resistance (NO) to lysis by various groups of clones. Note that loss of expression of the paternal or maternal HLA-A allele (as in variants 81B2 and 81TA) does not modify the resistance to lysis. Variant 81OA, which lost expression of all class I (but not class II) antigens, became susceptible to lysis by all groups of clones. On the other hand, loss of class I antigens coded for by the paternal chromosome 6 (81G) resulted in susceptibility to lysis by clones specific to groups 1 and 5. Conversely, a variant selectively lacking class I antigens coded for by the maternal chromosome 6 (81TB) became susceptible to lysis by group 2 clones (but not by group 1 or 5).

type. These studies indicated that genes that regulate susceptibility or resistance to lysis by group 1 alloreactive clones map in the MHC region between Bf (complement cluster) and the HLA-A locus, thus encompassing the class I loci HLA-B and HLA-C (59). To identify the genes encoding the molecules recognized by alloreactive NK clones, we used as a source of target cells an Epstein–Barr virus (EBV)-transformed B cell line derived from a donor who was heterozygous for three distinct NK-defined specificities (defined by clone groups 1, 2, and 5, respectively) (60). Five variants derived from this cell line (see Fig. 2), which lacked surface expression of one or more HLA class I molecules, were analyzed. As shown in Fig. 2, B2 and TA variants selectively lack the paternal or the maternal HLA-A alleles, respectively. G and TB variants lost all the MHC alleles encoded by either the paternal or the maternal chromosome 6, respectively, Finally, the OA variant had lost the ability to express all MHC class I genes, but it expressed all class II molecules. Analysis of susceptibility to lysis by NK clones displaying different specificities showed that B2 and TA variants were still resistant to lysis by clones belonging to groups 1, 2, and 5. On the other hand, the G variant was killed by group 1 and 5 clones. The TB variant, on the contrary, was killed by group 2 clones. Finally, the OA variant was lysed by all groups of NK clones (Fig. 2). These data strongly suggested that distinct MHC class I alleles (with the exception of HLA-A) may be responsible for "protection" of target cells from NK cell-mediated lysis (at least for the specificities analyzed).

The direct demonstration that HLA class I alleles may indeed function as protective elements against NK cells was provided by the analysis of murine P815 cells transfected with different human class I alleles (60). We focused our attention on the Cw3 allele because it was absent in variants that acquired susceptibility to lysis by group 2 clones. In addition, in representative families, Cw3 cosegregated with the dominant allele, conferring resistance to lysis by group 2 NK clones (i.e., all individuals who inherited the Cw3 allele were resistant to lysis by group 2 clones).

Indeed, experiments in which murine P815 cells transfected with Cw3 have been used as target cells demonstrated that these cells were resistant to lysis by group 2 clones. On the contrary, P815 cells, either untransfected or transfected with other HLA class I alleles (A2, A3, and A24), were highly susceptible to lysis by group 2 clones. The specific protective effect of the Cw3 allele was further substantiated by the finding that clones belonging to other groups (for example, group 1 clones), efficiently lysed Cw3-transfected cells (Fig. 3). As discussed

earlier, resistance to lysis by group 2 NK cell clones in informative families cosegregated with the Cw3 allele and also with Cw1 and Cw7 alleles. Interestingly, these HLA-C alleles share amino acids at positions 77 (serine) and 80 (asparagine) in the putative peptide-binding site of HLA-C (61). A possible interpretation is that this epitope may be involved in the mechanism of protection against group 2 NK clones. In this context Storkus *et al.* (62) identified an epitope characterized by an asparagine at position 74, shared by HLA-A3, A11, A24, Aw68, Aw69, B7, and B27, which appears to confer protection from lysis mediated by fresh polyclonal NK cells. Further analysis of other HLA-C alleles indicated that Cw4 acts as a protective element for group 1 clones. This has been shown by the use of the C1R cell line, which expresses Cw4 as the only serologically detected class I molecule (63, 64). C1R cells were efficiently lysed by clones displaying various specificities with the exception of group 1 clones (Fig. 3).

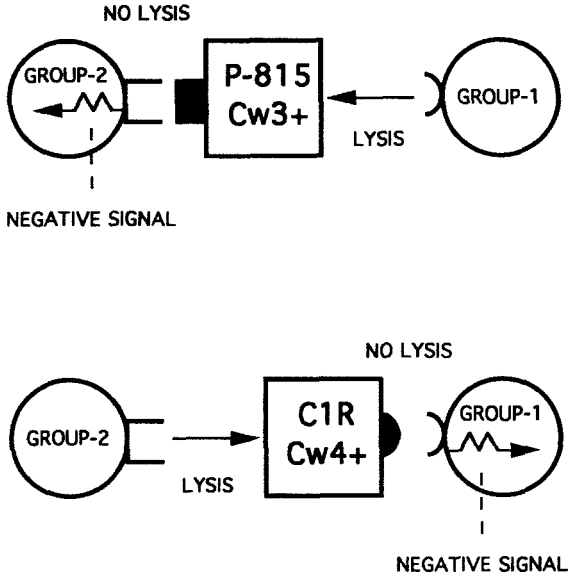

FIG. 3. Expression of HLA-C alleles specifically protects from lysis mediated by NK clones of defined specificity. (Top) A $Cw3^+$ target cell (the murine P815 cells transfected with the human HLA Cw3 gene) is not lysed by group 2 NK clones, but is efficiently lysed by group 1 clones. (Bottom) A $Cw4^+$ target cell (the human C1R cell line, which expresses HLA Cw4 as the only detected class I allele). C1R is resistant to lysis by group 1 clones but is efficiently lysed by group 2 clones. As discussed in the text, a likely explanation for the selective protection mediated by class I alleles is that interaction of class I alleles with clonally distributed specific receptors is responsible for a "negative" signal that prevents NK-mediated target cell lysis.

Again, analysis of representative families suggested that other HLA-C alleles could function as protective elements against lysis of anti-1 clones (48). Again, these alleles, including Cw2, Cw5, and Cw6, shared with Cw4 two amino acids at positions 77 and 80 (asparagine and lysine, respectively) (61).

It is worth noting in our experiments that, so far, at the clonal level we have evidence for involvement of HLA-C alleles, whereas we are unable to confirm involvement of HLA-A alleles as protective elements for given groups of NK clones. The discrepancies with data obtained by Storkus *et al.* (62), in polyclonal NK cell populations, may reflect technical differences, including the type of NK cells utilized (polyclonal versus clonal), or differences in the MHC haplotype of the donors analyzed.

It is certain that experiments performed in our and other laboratories provide suggestive evidence that class I molecules represent the protective element against lysis by NK cells. Further evidence for the direct involvement of class I molecules was recently obtained by the use of an antibody directed against some HLA-C alleles (termed 12B4). This antibody has been selected on the basis of the ability to restore lysis of C1R cells by group 1 clones and it is likely to be directed against epitopes of class I molecules involved in the protective effect. The use of 12B4 mAb [or its $F(ab)_2$ fragment] has allowed us to establish that class I molecules are indeed the direct molecular target of NK cell recognition in group 1 clones. In addition, using this new MAb, it has been possible to establish that class I molecules represent the protective element in NK clones that do not display the ability to kill normal allogeneic cells (see earlier). Accordingly, these clones did not lyse $Cw3^+$ P815 cells, $Cw4^+$ C1R cells, or PHA blasts of the panel shown in Table I. The ability of the 12B4 mAb to induce lysis of "protected" target cells provides evidence for a direct involvement of class I molecules in this case. A possible interpretation is that these clones may recognize a nonpolymorphic determinant expressed by C alleles.

A most important fact provided by the 12B4 mAb was that class I molecules are directly involved in the mechanism of protection from autologous NK cells. Thus, for example, in a $Cw4^+$ donor, addition of 12B4 mAb induced lysis of normal autologous cells by (autologous) group 1 clones. This strongly suggests that self class I molecules may act as elements that specifically protect normal cells from NK-mediated autoreactivity. As we shall discuss later, conditions that lead to loss (or masking) of class I molecules (or alleles) result in NK-mediated lysis of autologous cells (58, 65–68).

VIII. Clonally Distributed Molecules (p58) Involved in Regulation of NK-Mediated Cytolysis

The evidence that human NK cells are able to recognize MHC class I alleles and that this function is clonally distributed implies that distinct surface receptors, mediating specific recognition, exist in different NK cells. The approach applied to the identification of these receptors has been based on the selection of mAbs reacting with clonally distributed (or NK subset-specific) surface molecules. After immunization of mice with $CD3^-16^+$ alloreactive clones we selected two mAbs, termed GL183 (69) and EB6 (70), that reacted with surface molecules that are selectively expressed on the surface of human NK cells. The two molecules recognized by GL183 and EB6 mAbs were expressed on two partially overlapping subsets of NK cells. In addition, they were present in both resting and activated NK cells and their expression (or lack thereof) was not changed by cell activation, proliferation, or cloning. The phenotypic stability of GL183 and EB6 expression allowed definition of four distinct NK subsets: $GL183^+EB6^+$, $GL183^+EB6^-$, $GL183^-EB6^+$, and $GL183^-EB6^-$ (see Fig. 4) (70). Biochemical analysis of molecules immunoprecipitated by the two mAbs indicated that the antigenic determinants recognized by EB6 and GL183 mAbs are carried on distinct chains (70). Further peptide map analysis of GL183 or EB6 molecules immunoprecipitated by phenotypically different NK cell clones showed that, although the two molecules share major peptides, unique peptides in each molecule could be identified. The findings that the apparent molecular weight as evaluated by sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) is identical for EB6 and GL183 and that the two molecules share a majority of peptides in peptide mapping strongly suggest that the two molecules are highly homologous (belonging to a new NK-specific molecular family) (70). It has been shown that both EB6 and GL183 molecules are noncovalently associated to the CD3 ζ chain (43) and to the γ chains of FcεRI. Thus, because molecules involved in signal transduction are associated with p58 molecules, this supports the concept that p58 molecules may serve as surface receptors in NK cell subsets.

Functional analyses indicated that both GL183 and EB6 mAbs, in a redirected killing assay using the $Fc\gamma R^+$ P815 target cells and mAb-reactive NK clones as effectors, inhibited target cell lysis (69–71). Not only did anti-p58 mAb inhibit the spontaneous lysis of P815 cells, but also the lysis of P815 induced by anti-CD16 or anti-CD2 mAb or PHA. In apparent contradiction to the inhibitory effect against P815 target cells, both GL183 and EB6 mAbs increased the cytolytic activity of

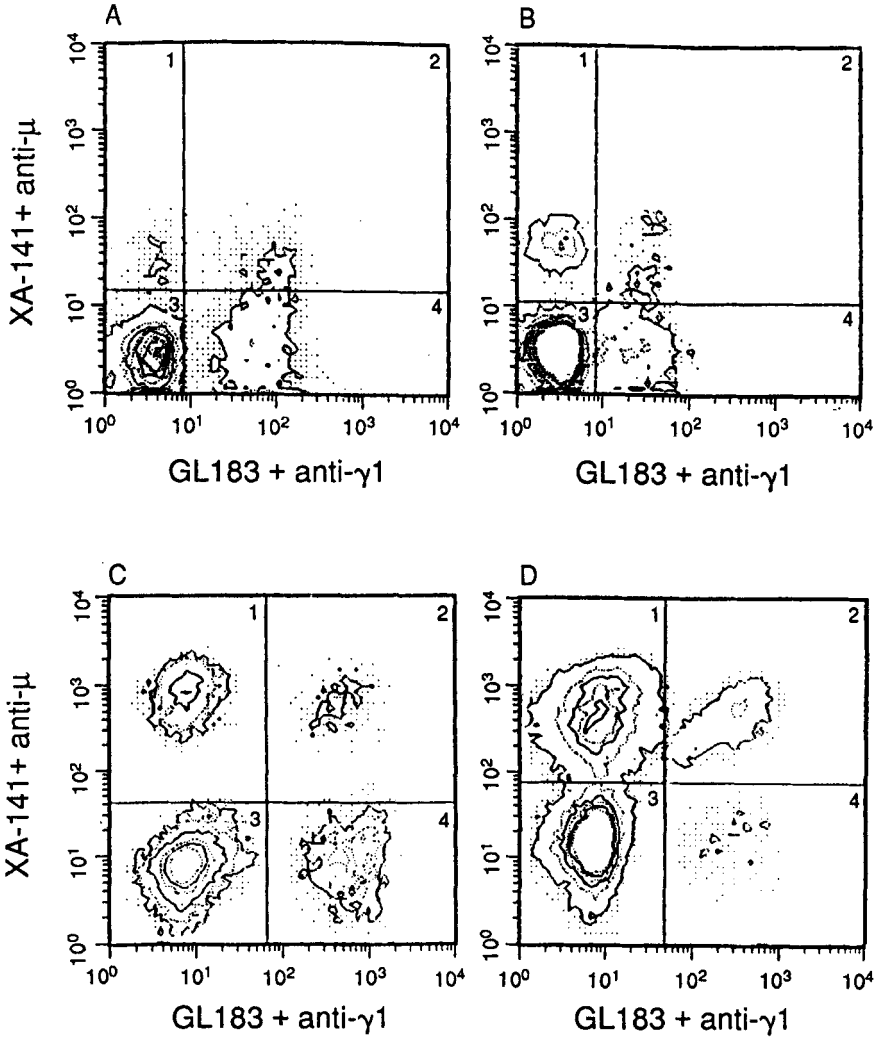

FIG. 4. Four subsets of human NK cells can be identified on the basis of the expression of GL183/EB6 molecules. In these experiments, the surface expression of GL183 and EB6 molecules has been evaluated by double fluorescence and fluorescence-activated cell-sorting analysis of both peripheral blood lymphocytes and activated $CD3^-CD16^+$ "bulk" cell populations. Cells were stained with GL183 mAb followed by a second reagent represented by a goat antimouse IgG_1 antiserum (fluorescein isothiocyanate conjugated) and by the XA-141 mAb (anti-EB6) followed by a second reagent consisting of an anti-IgM phycoerythrin. The contour plot is divided into four quadrants representing unstained cells (lower left), cells with only red fluorescence (upper left), cells with red and green fluorescence (upper right), and cells with only green fluorescence (lower right). A and B represent the peripheral blood mononuclear cells derived from two distinct donors, whereas in C and D two "bulk" populations consisting of activated $CD3^-CD16^+$ cells were evaluated. It is evident that the percentage of cells belonging to the various cell subsets varied in the four subjects analyzed.

human tumor cell lines resistant to NK clones. A likely explanation for this phenomenon will be provided in Section IX.

Given the involvement of p58 molecules in target cell lysis, we investigated whether a correlation existed between the GL183/EB6 phenotype and the ability of NK clones to recognize "protective" class I alleles. A remarkable correlation was found between the EB6/GL183-defined surface phenotype and the allospecificity mediated by a given group of clones (51, 70) (Fig. 5). For example, in most individuals, all NK clones characterized by the $EB6^+GL183^-$ surface phenotype belong to group 1 (in which the protective element is represented by Cw4 or related alleles). Importantly, in individuals susceptible to lysis by group 1 clones, the $EB6^+GL183^-$ subset was virtually absent (70). In addition, in these susceptible individuals, the rare $EB6^+GL183^-$ clones [group 5 clones (51, 60)] were unable to kill autologous cells. These findings are clearly consistent with the existence of tolerance and/or clonal deletion in the NK cell populations. Analysis of the correlation existing between the GL183/EB6 surface phenotype and the specificities of NK clones clearly indicates that all other groups of alloreactive clones are characterized by a homogeneous GL183/EB6 phenotype. These data are in support of the hypothesis that the p58 molecular family is involved in the specific recognition of class I molecules by NK cells.

IX. p58 Molecules as Putative Receptors for MHC Class I Molecules in Human NK Cells

Available data suggest that, in contrast to T lymphocytes, specific recognition of MHC class I molecules in NK cells induces inhibition of cytolytic activity and thus "protects" target cells (51, 60). In view of the precise correlation established between the expression of GL183 and EB6 surface molecules and the specificity of NK clones, the possibility that p58 molecules could represent the specific receptor for MHC class I was directly assessed by experiments in which anti-p58 mAbs were used as inhibitors (72). If p58 molecules represent the receptor for MHC class I, anti-p58 mAbs should interfere with the p58/class I interaction.

As discussed above, NK clones displaying "specificity 1" and expressing the $GL183^-EB6^+$ phenotype failed to lyse $Cw4^+$ target cells. Addition of the EB6 mAb [or its $F(ab)_2$ fragment] induced lysis of Cw4-protected target cells (72). Similarly, in $GL183^+EB6^+$ clones belonging to group 2 (in which the protective element is represented by Cw3), both GL183 and EB6 mAbs [or the relative $F(ab)_2$ fragments]

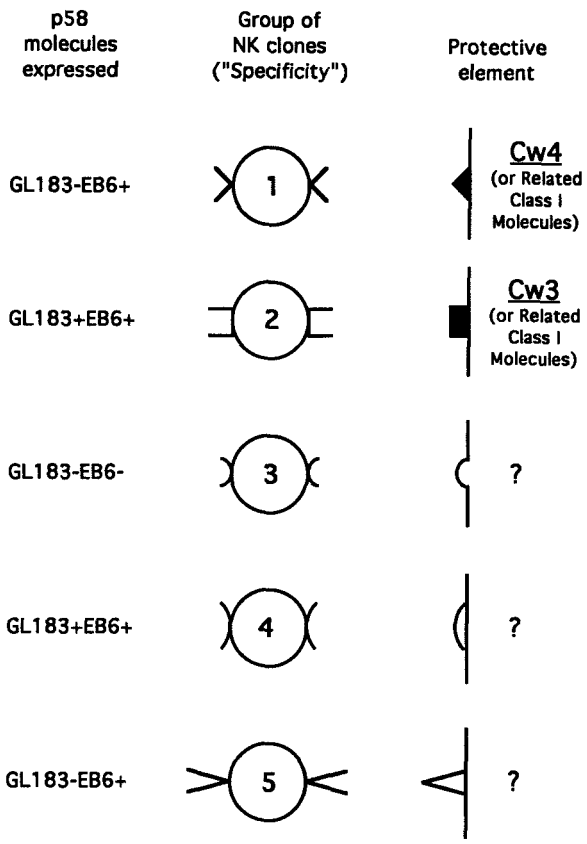

FIG. 5. The human NK cell repertoire. Human NK cells express a clonally distributed ability to lyse selectively normal allogeneic cells (see Table I). Cw3 and Cw3-related MHC class I molecules function as a protective element that selectively prevents target cells from lysis by group 2 clones. In addition, Cw4 (and related alleles) may function as a protective element for group 1 clones. According to this interpretation, the ability of a given group of clones to lyse selectively only certain allogeneic cells would be determined by the lack of expression, on these cells, of different specific protective elements. The clonally distributed surface receptors expressed by different groups of alloreactive NK cell clones would recognize different protective elements. Five distinct allospecificities have been identified so far, but the protective elements for clones of groups 3, 4, and 5 are still unknown. Moreover, each group of alloreactive clones is confined to an NK cell subset that can be identified on the basis of the differential expression of functional surface molecules that can be detected by the mAbs GL183 and EB6; these molecules belong to a novel NK-specific 58-kDa molecular family and appear to compose, in different association, the NK receptor for class I molecules (see Figs. 6–11 and the text).

induced lysis of Cw3-protected target cells (72) (Fig. 6). A likely interpretation of these data is that the reconstitution of lysis of protected cells is due to masking of the NK receptors for class I molecules by anti-p58 mAbs. According to this interpretation, the finding (described in Section VIII) that anti-p58 mAbs are able to increase lysis of most human tumor target cells may be interpreted again as a masking effect, which would prevent NK receptors from interacting with protective class I molecules expressed by allogeneic tumor cells. Thus, p58 molecules appear to be part of the class I-specific NK receptor, which, on binding with class I molecules, delivers a negative signal to the NK cells. In agreement with this concept, triggering of NK cells with entire IgG anti-p58 mAbs cross-linked by $Fc\gamma R^+$ target cells (in a redirected killing assay; see above and Fig. 7) results in strong inhibition of cytolytic activity. In these experiments, the cross-linked entire anti-p58 mAbs would mimic the effect of class I molecules expressed on protected target cells (69–72).

X. Human NK Receptors for Class I Molecules Are Composed of p58 Dimers

In clones expressing the $GL183^+EB6^+$ phenotype and specific for the Cw3 allele (60), reconstitution of lysis can be equally achieved with GL183 or EB6 mAb (72). A likely interpretation of this is that both p58 molecules participate in the recognition of the specific protective element. For example, the NK receptors in this group of clones could be formed by the association of GL183 and EB6 molecules. Antibody-induced modulation of EB6 surface molecules (72a) demonstrated that GL183 molecules undergo specific comodulation (Fig. 8). Further evidence for a molecular association was provided by biochemical analysis of p58 molecules. When, $GL183^+EB6^+$ clones were lysed under conditions that preserve the molecular association (i.e., digitonin), both GL183 and EB6 molecules were coimmunoprecipitated by either of the anti-p58 mAbs. This suggests that two p58 chains are noncovalently associated to form heterodimers at the NK cell surface. In the case of $GL183^-EB6^+$ NK clones, our data suggest that the differential association of p58 molecules determines the specificity of the NK receptors. Thus the EB6 molecule, when associated with GL183, recognizes Cw3 (or related alleles), whereas in the absence of GL183 ($GL183^-EB6^+$ clones), Cw4 or related alleles are recognized. However, for $GL183^-EB6^+$ clones it is necessary to hypothesize the existence of EB6-associated molecules. Indeed, if the single EB6 molecule is sufficient to recognize a class I ligand, all the cells bearing EB6 (including $GL183^+EB6^+$) would always recognize the same alleles

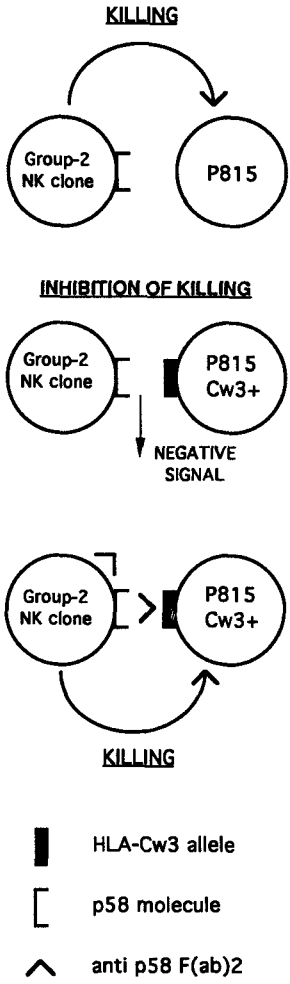

FIG. 6. Antibody-mediated "masking" of p58 molecules restores the lysis of class I-"protected" target cells. As describe in the text, group 2 NK clones ($GL183^+EB6^+$) lyse target cells that lack expression of Cw3 or related alleles (e.g., untransfected P815 cells). Expression of Cw3 on target cells (consequent to transfection with the human HLA Cw3 gene) results in protection from lysis mediated by group 2 clones. Adding $F(ab)_2$ fragments of p58 mAbs (either GL183 or EB6) results in complete restoration of lysis of $Cw3^+$ cells. These results can be interpreted as a masking effect mediated by the $F(ab)_2$ fragments, which prevent the interaction of p58 molecules with their class I ligand on target cells.

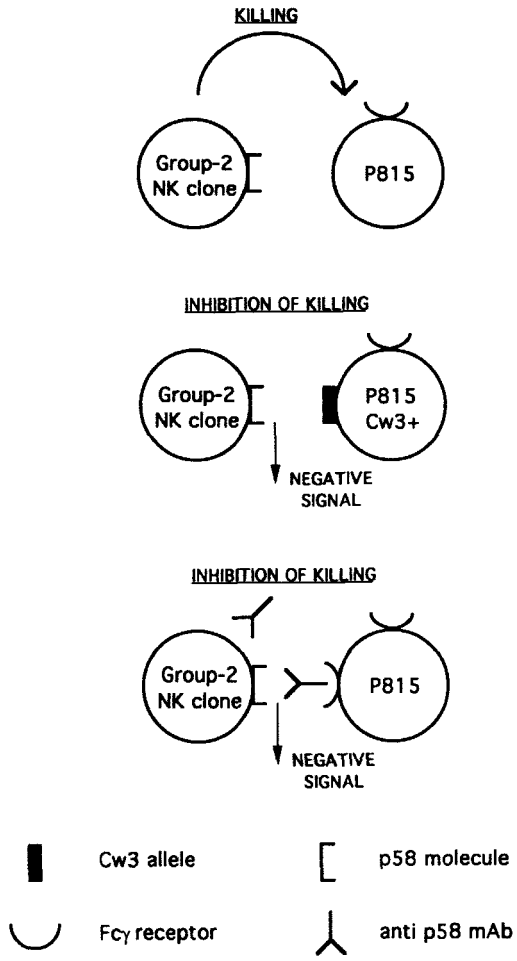

FIG. 7. Anti-p58 mAbs mimic the inhibitory effect of class I molecules on the NK-mediated cytolysis. The figure refers to a group 2 NK clone (expressing both GL183 and EB6 molecules), as effector, and to the murine P815 cells transfected or not with Cw3 (the protective element for group 2 clones), as targets. The group 2 NK clones efficiently lyse untransfected P815 cells, but not P815 cells transfected with Cw3. When the entire GL183 or EB6 mAbs (which bind via their Fc portion to the $Fc\gamma R^+$ P815 cells) are added, inhibition of lysis of untransfected P815 cells occurs. Thus, it appears that the anti-p58 mAbs bound on target cells protect them from lysis, i.e., they mimic the effect of Cw3 molecules in delivering an inhibitory signal on cross-linking of the p58 molecules expressed by group 2 clones.

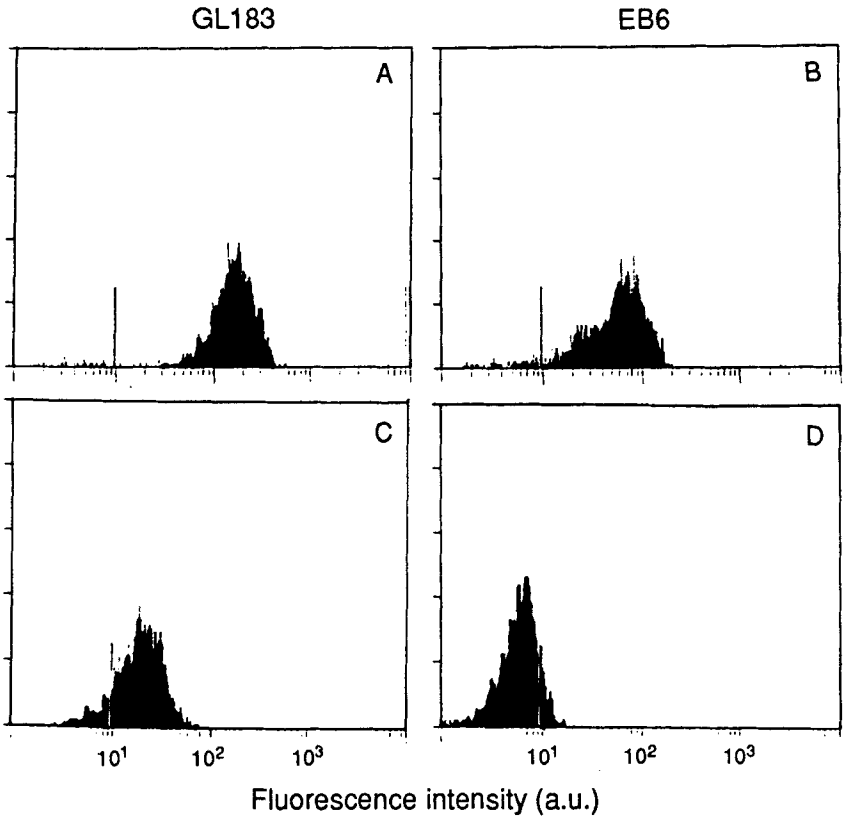

FIG. 8. Comodulation of GL183 and EB6 molecules in GL183$^+$EB6$^+$ NK clones. In this experiment, a GL183$^+$EB6$^+$ NK clone was cultured for 18 hours in costar wells coated with the XA-141 mAb (specific for EB6 molecules). Subsequently, cells were washed and analyzed by indirect immunofluorescence for GL183 and EB6 expression. (A) Unmodulated cells stained with GL183 mAb. (B) Unmodulated cells stained with EB6 mAb. (C) XA-141-modulated cells stained with GL183 mAb. (D) XA-141-modulated cells stained with EB6 mAb. It is evident that a complete modulation of EB6 molecules resulted in comodulation of most GL183 molecules.

(i.e., Cw4 or related alleles). Two possibilities exist for the composition of the NK receptor expressed by Cw4-specific GL183$^-$EB6$^+$ clones: (1) EB6 molecules are associated with a still undefined p58 molecule; and (2) EB6 molecules are associated with other EB6 molecules, forming homodimers (Fig. 9). Although the specificity of GL183$^-$EB6$^+$ clones for Cw4 is unquestionable in several individuals tested, in two individuals GL183$^-$EB6$^+$ clones did not recognize Cw4 or related alleles (the previously defined group 5) (51). It is likely that

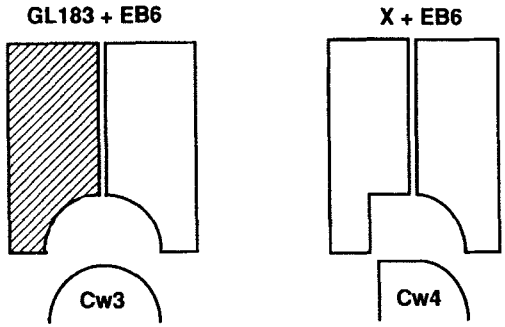

FIG. 9. Association of different p58 molecules determines the specificity of the NK receptors for different class I molecules. The differential association of p58 molecules determines the specificity of the resulting noncovalently linked heterodimers. This is a schematic representation of the molecular association of different p58 molecules in NK clones of known specificity. (Left) Group 2 NK clones coexpress GL183 and EB6 molecules and recognize Cw3 or related alleles. (Right) Group 1 clones express EB6 and a still undefined p58 molecule. This association is responsible for the recognition of Cw4 or related alleles. Note that it cannot presently be excluded that p58 molecules may instead form homodimers; for example, the $GL183^-EB6^+$ clones could actually be represented by homodimers of EB6 molecules ($EB6^+EB6^+$).

in these individuals EB6 molecules may be associated, forming dimers different from those of Cw4-reactive group 1 clones. Differences in the specificity of $GL183^-EB6^+$ clones isolated from different individuals appear to be related to the HLA haplotype of these individuals. Thus, individuals in which $GL183^-EB6^+$ clones recognize Cw4 or related alleles are characterized by an HLA haplotype containing the Cw4 (or related) allele, but this is not the case for donors in which $GL183^-EB6^+$ clones belong to group 5.

XI. Effect of p58 Modulation on Cytolytic Activity

Since p58 molecules appear to function as NK receptors for class I molecules, we analyzed the effect of removal of p58 molecules from the NK cell surface as a consequence of mAb-induced modulation. Modulation of EB6 molecules in either $GL183^+EB6^+$ or in $GL183^-EB6^+$ clones specific for Cw3 and Cw4 alleles, respectively, resulted in lysis of Cw3- or Cw4-protected target cells (72a). These data further support the notion that dimers of p58 molecules represent the NK receptors specific for MHC class I alleles, and that, in the absence of such receptors, MHC-specific NK cells became unable to distinguish between protected and unprotected target cells. In line

with this view, the inability to kill protected target cells was recovered only after reexpression of p58 molecules (Fig. 10).

Taken together, these data indicate not only that p58 molecules represent the putative receptors for class I molecules, but also that the specificity for different HLA-C alleles is determined by the differential association of different members of the p58 molecular family.

XII. Opposite Effect of Specific MHC Recognition by T Lymphocytes or NK Cells

Two alternative hypotheses have been proposed to explain MHC recognition by NK cells: (1) the "target interference" hypothesis, in which it is postulated that class I MHC molecules and/or their associated peptides mask recognition of the actual NK target structure responsible for NK cell activation, and (2) the hypothesis that implies the delivery of a regulatory negative signal to NK cells after interaction with the "protective" MHC molecule. These two alternative hypotheses have been discussed in detail in previous reviews (58, 65–68). All

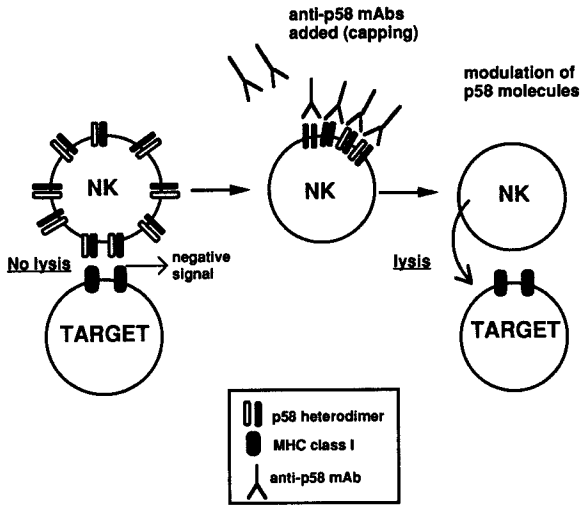

FIG. 10. Effect of removal of p58 molecules by antibody-induced modulation. In NK clones of defined specificity (e.g., Cw3-specific group 2 clones that coexpress GL183 and EB6 molecules), mAb-induced modulation of EB6 molecules results in comodulation of GL183 molecules. As a consequence, group 2 clones lose the ability to recognize Cw3 molecules (which act as a protective element for group 2 clones) and thus kill the $Cw3^+$ target cells.

of our data are compatible with the second hypothesis. This hypothesis requires the following conditions:

1. The expression of specific class I alleles on protected target cells.
2. The expression of receptors specific for class I alleles on NK cells.
3. The ability of this receptor to deliver inhibitory or inactivating signals on interaction with specific MHC molecules.
4. The existence on NK cells of surface molecules capable of mediating NK cell activation following binding with (non-MHC-encoded) ligands expressed on target cells.

The interaction between specific receptors and MHC-encoded surface molecules appears to be a central event regulating the cytolytic function of both NK and T lymphocytes. However, whereas in T cells TCR/MHC interaction induces a positive signal resulting in triggering of cytolysis (73), in NK cells the NK receptor/MHC interaction would induce a negative signal resulting in inhibition of cytolytic activity. This opposite functional effect can also be elicited by antireceptor mAbs. Thus, anti-TCR mAbs trigger the cytolytic activity of alloreactive CTLs in a redirected killing assay (74), thus mimicking the effect of alloantigen recognition ("antigen-like" effect) (75, 76). Opposite to anti-TCR, anti-p58 mAbs under the same experimental conditions strongly inhibit the NK-mediated cytolysis, thus mimicking the protective effect of MHC molecules (69–72). On the other hand, mAb-mediated masking of TCRs, by preventing MHC recognition, inhibits specific lysis of allogeneic cells (73), whereas masking of p58 molecules, by preventing MHC recognition, would allow lysis of MHC-protected target cells by inhibiting the delivery of the negative signal (72).

Opposite functional effects can also be detected after mAb-induced modulation of TCRs or NK receptors. Thus, modulation of TCRs in alloreactive cytotoxic T lymphocytes (CTLs) prevents alloantigen recognition (77) and, as a consequence, lysis of specific target cells. On the contrary, modulation of NK receptors for the MHC in class I-specific NK cells prevents MHC recognition and results in lysis of protected target cells (72A) (Fig. 11).

XIII. The Putative Murine NK Receptor for the MHC

An inhibitory receptor specific for MHC class I molecules expressed on target cells in mice has been identified. This molecule is represented by Ly49, which is expressed at the cell surface as a disulfide-

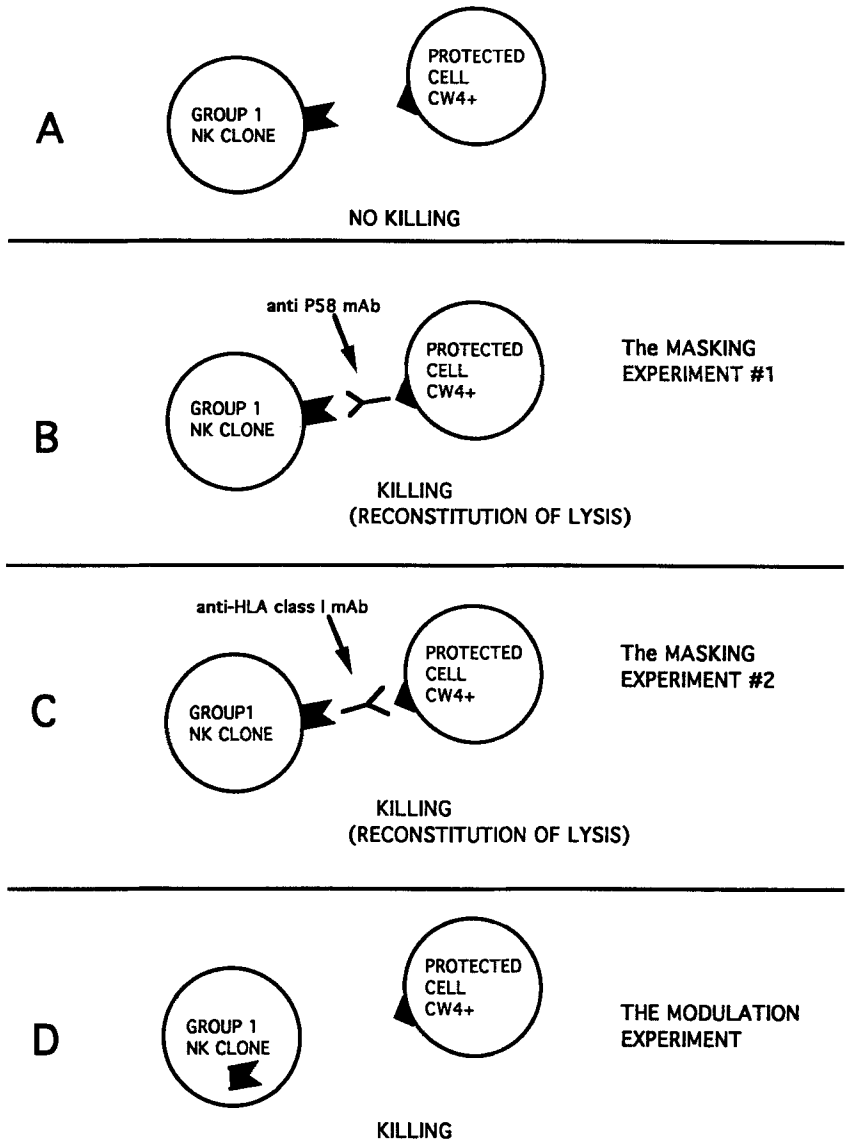

FIG. 11. Evidence that NK clones do not lyse class I-"protected" target cells as a consequence of the interaction between NK receptors and class I alleles. (A) A group 1 NK clone specific for Cw4 (or related C alleles) does not lyse $Cw4^+$ cells. Note that this situation also occurs, for example, in autologous combinations, i.e., in $Cw4^+$ individuals who have a fraction of their NK cells specific for Cw4. (B) Interaction between p58 and Cw4 is blocked by anti-p58 mAbs. The mAb-mediated masking of the NK receptor results in lysis of Cw4-protected target cells. (C) Interaction between p58 and Cw4 is blocked by an appropriate anti-class I mAb (12 B4) specific for C alleles. Also, masking of Cw4 results in lysis of Cw4-protected target cells. (D) Removal of p58 receptors by mAb-induced modulation prevents recognition of Cw4 molecules on target cells. This results again in lysis of Cw4-protected target cells.

linked homodimer formed by 44-kDa subunits (78, 79). The Ly49 antigen is expressed by a subpopulation (~20%) of splenic NK cells (80) and, more importantly, in H-2^b strains (81, 82) the Ly49$^+$ and Ly49$^-$ subsets have been shown to differ in their specificity. Ly49$^+$ NK cells do not lyse H-2^d and H-2^k tumor target cells, whereas Ly49$^-$ NK cells do (83). Transfection experiments demonstrated that the protective element specifically recognized by Ly49$^+$ cells is represented by H-2D^d. Similar to anti-p58 mAbs, anti-Ly49 antibodies can reverse resistance to lysis. In addition, similar to the anti HLA-C mAb selected in our lab (12B4 mAb) (83a), antibodies directed to the $\alpha 1/\alpha 2$ domains of H-2D^d rendered protected target cells susceptible to lysis mediated by Ly49$^+$ cells (83). An apparent paradox in these studies is represented by the finding that allogeneic H-2^d tumors were protected from lysis by Ly49$^+$ cells derived from H-2^b mice whereas syngeneic tumors cells were lysed. Moreover, syngeneic (H-2^b) normal cells were protected from lysis. A possible interpretation of these data is that, in H-2^b mice, Ly49$^+$ cells do not recognize self MHC molecules, but rather allogeneic MHC. Their ability to lyse syngeneic tumor cells but not normal cells cannot be explained by a mechanism of protection based on the MHC haplotype because both cells apparently expressed the same H-2^b molecules (83).

Two possible explanations could be envisaged for these results: (1) Normal and tumor cells differ in surface expression of ligands able to interact with "activatory receptors" expressed by Ly49$^+$ cells (see also Section XV). (2) Syngeneic MHC elements may be protective for normal cells, whereas in tumor cells the protective MHC element could be either missing or masked (e.g., by tumor peptides). The latter interpretation may explain a major difference between the murine and human system. Thus, in humans the protective elements were invariably represented by self and self-related class I alleles. On the contrary, in the mouse, only allogeneic MHC molecules have been described as protective elements for the Ly49$^+$ cells. At the present time, the role of Ly49$^+$ cells as effector cells specific for allogeneic (but not for syngeneic) protective class I molecules is difficult to interpret in a physiological context.

XIV. Other Possible Murine Receptors and the Hybrid Resistance Phenomenon

Another murine surface molecule that could serve as an NK receptor is represented by a disulfide-linked dimer (subunits of 54 kDa) that is expressed by approximately 50% of NK cells and is recognized by the

mAb SW5E6 (84). NK cells expressing these molecules mediate rejection of bone marrow cells expressing H-2^d/Hh-1^d in what is termed the hybrid resistance phenomenon. The hybrid resistance phenomenon (85) refers to the situation in which parental bone marrow is rejected by NK cells from lethally irradiated F_1 hybrid mice. This phenomenon, which cannot be explained on the basis of conventional transplantation laws, was described more than 20 years ago by Cudkowicz (86–89). The genes, which encode the antigens involved in the hybrid resistance on donor bone marrow cells, the so-called hematopoietic histocompatibility (Hh-1) antigens, have been mapped to the MHC region near the *H-2D* locus (90). Bone marrow cells derived form H-2^b mice, transgenic for H-$2D^d$, are able to engraft (H-$2D^b$ × H-$2D^d$)F_1 hybrid mice (56), compatible with the possibility that the MHC class I locus, *H-2D*, is inversely responsible for hybrid resistance.

A possible interpretation, based on our data in the human system, could be that parental bone marrow cells are rejected in F_1 hybrid mice because the donor cells lack half of the MHC molecules expressed by the F_1 recipients.

XV. "Receptors" Mediating NK Cell Activation

Since receptors for class I molecules appear to deliver negative signals, resulting in inhibition of cytolytic activity, the existence of receptors that mediate NK cell triggering responsible for lysis of susceptible target cells must be postulated (59, 60, 72). Thus, interaction between activatory receptors and their ligands on target cells would result in classical non-MHC-restricted lysis (66). On the other hand, the concomitant interaction of the NK receptor for MHC and protective class I molecules would down-regulate the function of activatory receptors (66, 72).

In humans, several molecules have been shown to induce NK cell activation. Thus, CD16, which binds with low affinity the Fc portion of IgG, can mediate NK cell triggering on binding IgG immunocomplexes (1, 91). Another surface molecule, the CD2 molecule, is expressed by resting or activated NK cells and mediates cell triggering and target cell lysis (in a redirected killing assay) (92–94). Activated NK cells (i.e., LAK cells, including NK clones) display an antitumor cytolytic activity that is severalfold higher than that of resting NK cells. In this respect, another triggering molecule expressed by activated but not resting NK cells, i.e., the CD69 molecule, may play a relevant role in triggering the cytolytic activity of activated NK cells (95).

If one speculates that a single type of activatory receptor is responsi-

ble for inducing non-MHC-restricted lysis of unprotected target cells, neither CD16 nor CD2 would represent suitable candidates for this role. Indeed, NK cells that lack either antigen can still mediate cytotoxicity (95–97). Also, CD69 cannot be seen as the unique activatory receptor for NK cells, because, although expressed and functional on all activated NK cells, it is absent on most resting NK cells (95).

Two hypotheses are still possible to explain the mechanism of NK cell activation by ligands expressed on nonprotected target cells: (1) an unidentified activatory receptor responsible for the MHC-unrestricted lysis, and (2) that MHC-unrestricted lysis is the result of multiple interactions involving different activatory receptors and their ligands on unprotected target cells.

In favor of the existence of a unique receptor, recent studies in the rat and in the mouse suggested a critical role in NK cell activation of a new family of membrane proteins composed of disulfide-linked homodimers. These molecules, termed NKR-P1 (30-kDa subunits in the rat), are expressed on all rat NK cells, are type II integral membrane proteins (with the C terminus exposed extracellularly), and are homologous to members of the C-type lectin superfamily (98, 99). In the mouse, the NK1.1 antigen shares several similarities with NKR-P1 and has been shown to belong to the same molecular family (100–102). It is possible that this molecular family may represent the unique activatory receptor for rat and mouse NK cells.

Consistent with this hypothesis, mAb 3.2.3 (antirat NKR-P1) (103, 104) triggers the NK-mediated cytolytic activity in redirected killing assays, stimulates release of serine esterases, and activates phosphoinositide turnover. Also, mAb PK136 (antimouse NK1.1) displays similar abilities to modify NK cell functions (105). These functional capabilities, together with the finding that these molecules display some degree of polymorphism (100), suggest that these molecules may represent an NK receptor responsible for activatory signals in the killing of unprotected target cells. However, it is likely that NKR-P1 does not represent the unique activatory receptor responsible for natural killing (106). Thus, for example, mAbs against these molecules were unable to block lysis of unprotected target cells. This may indicate that NKR-P1 represents one of the molecules involved in NK cell activation, but not the unique receptor. Thus, based on our current knowledge, activation of NK cells by unprotected target cells would appear to involve multiple receptor/ligand interactions rather than unique activatory receptor. Another interesting functional molecule has been described in human NK cells by Lòpez-Botet *et al.* (107). This disulfide-linked 43-kDa molecule (Kp43) is expressed on virtually all NK cells and on a

small T lymphocyte subset. The Kp43 molecule is involved in the regulation of NK cell function since it modulates both their cytolytic activity and lymphokine production.

Finally, a family of five NK-specific genes has been recently characterized in humans. These genes, termed *NKG2*, have some degree of homology among them. However, the function and the cellular localization of the *NKG2*- encoded protein are still undefined (108, 109).

XVI. Concluding Remarks

In view of the new concepts on NK cells, what could the physiological role of NK cells be and how do NK cell function *in vivo*? Having established that, at least in humans, the molecular target for specific NK cell recognition is the (self) MHC class I molecules and that this interaction leads to inhibition of cytolysis (i.e., some class I alleles function as protective elements), several questions need to be asked.

Although, in general, class I molecules are expressed as a trimeric complex formed by the heavy chain, β_2-microglobulin, and bound peptide, it has not yet been clarified whether regulation of NK cell recognition requires all of these components or only one or two of them. In this context, Carbone *et al.* suggested that the β_2-free heavy chains expressed at the target cell surface are responsible for the mechanism of protection, at least from resting polyclonal NK cells (110). On the other hand, Storkus *et al.* emphasized the role of the class I-bound peptides. Thus, these authors showed that peptides specifically binding to the protective class I molecules could induce lysis of protected cells by polyclonal NK cells (111). Data are not yet available on the role of free heavy chains or peptides in the case of clones displaying a defined allele specificity.

Although the class I-mediated protection from NK cell lysis may represent a major mechanism of target cell resistance, this mechanism clearly requires further explanations and some exceptions should be considered.

Many tumor cells that are susceptible to lysis mediated by NK/LAK cells express normal amounts of surface class I antigens. This phenomenon can be explained in that (1) the tumor cells express class I antigens that do not function as specific protective elements for the allogeneic NK cells most frequently used in the cytolytic assays (Fig. 12) and (2) the tumor cells may lack the expression of single class I alleles functioning as protective elements (112, 113). It should be noted that conventional mAbs directed to common determinants of class I molecules do not allow detection of these class I allele-defective tumor

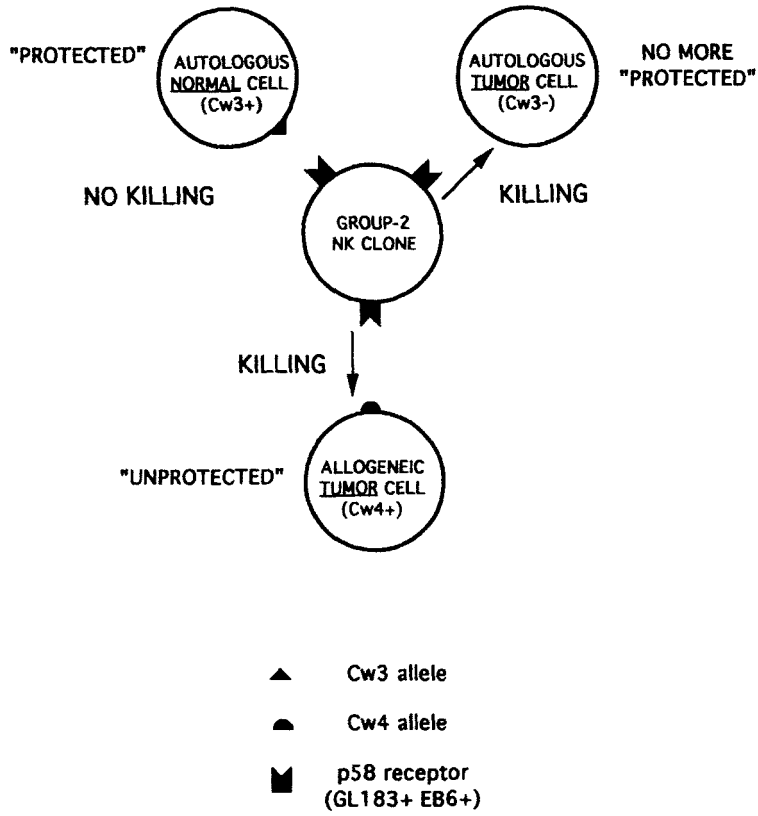

FIG. 12. NK cell-mediated lysis of autologous or allogeneic tumor cells. NK cells do not lyse normal autologous cells because they are protected by self class I molecules. In this case, a representative group 2 NK clone does not lyse autologous normal cells (PHA-induced blasts) because they are protected by surface Cw3 molecules. On the other hand, the same clone can lyse autologous tumor cells provided that Cw3 molecules either are not expressed or are masked (e.g., by tumor peptides). Allogeneic tumor cells can be lysed because they have lost the protective class I alleles or simply because they do not express specific protective alleles for group 2 clones (for example, the allogeneic tumor cell illustrated is $Cw4^+$).

cells. The use of allele-specific reagents shows that the loss of a given allele is a relatively common event in certain tumors.

Another possible mechanism of resistance to NK-mediated lysis may be related to the lack of appropriate interactions required for NK cell activation. Thus, target cells (either normal or tumor) that do not express class I molecules are not lysed by NK cells because they lack the

appropriate ligand(s) required for interactions with the activation receptor(s) expressed on NK cells. On the other hand, the failure of fresh, unactivated NK cells to lyse most tumor target cells may primarily reflect the lack of expression (or expression in low density) of the appropriate activation receptors. In this context, the ability of activated NK cells (i.e., LAK cells) to lyse efficiently a wider spectrum of tumor cells can be interpreted as the expression of appropriate activation receptors on these cells as a consequence of cell activation (Fig. 13) (114).

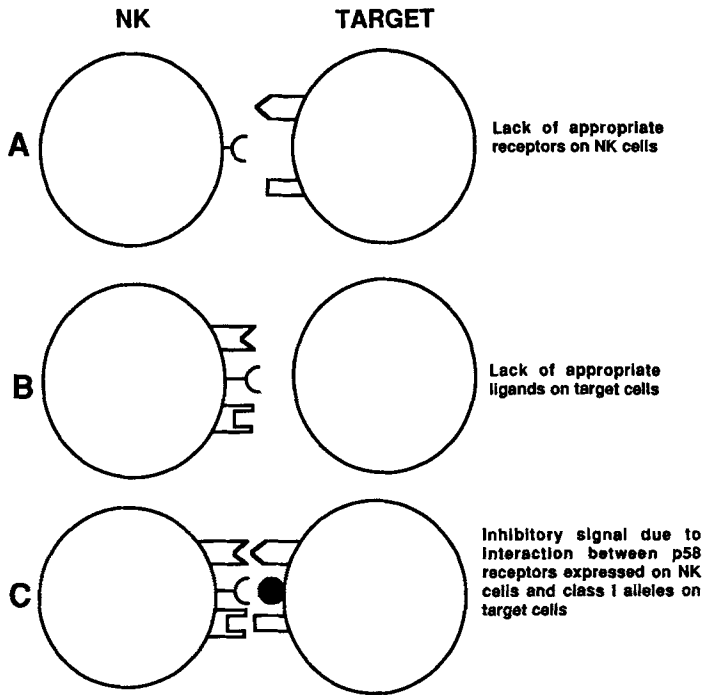

FIG. 13. Mechanisms by which target cells may be resistant to NK-mediated lysis. (A) NK cells lack appropriate "activatory" receptor molecules or express these receptors at low levels. This can occur in fresh, nonactivated NK cells. (B) Target cells lack appropriate ligands (for example, red blood cells are not lysed by NK cells). (C) In spite of efficient receptor/ligand interactions, target cells express appropriate class I alleles (●) that deliver an inhibitory signal via the p58 receptors expressed on NK cells.

How to explain the need for NK cells to recognize polymorphic self elements of MHC class I? A possible explanation can be deduced by the finding discussed above that some tumors lack the expression of single class I alleles (e.g., HLA-C). If NK cells play a role in the immunosurveillance against tumors, it is evident that NK cells equipped with clonally distributed receptors for polymorphic self alleles would have more chances to identify and lyse tumor cells. Possibly, a similar explanation could also be proposed for viral infection whereby NK cells have been shown to provide a first line of defense. In general, viral peptides are known to bind selectively to certain alleles [this would lead to masking of the protective epitope(s) that is part of the protective allele]. In all of these pathological conditions, the existance of NK cells specific for a self allele would represent a more effective mechanism of defense.

Regarding the possible mechanism by which NK cells have acquired their repertoire for different class I alleles, this could be the result of the rapid evolution of the MHC in terms of polymorphism. As a consequence, NK receptors have evolved to adapt to the progressive modifications of their MHC class I ligands.

Finally, most normal cells in an organism are protected from NK cell lysis because they express class I molecules. Since interaction between class I molecules and the specific NK receptors leads to NK cell inhibition, one may ask why NK cells continuously exposed to inhibitory signals (i.e., self MHC class I alleles) are still capable of lysing bystander, unprotected cells (e.g., tumor cells). Two possible explanations could be envisaged: (1) the inhibitory signal is short lived and (2) the inhibitory effect is limited to a portion of the cell and does not involve the whole cell. A polarized effect of the negative signal would not prevent NK cells from mediating cytolysis by utilizing other cell compartments. Preliminary data using bystander cell cytolytic assays are in favor of the second hypothesis (A. Moretta *et al.*, unpublished). In conclusion, NK cells play a role complementary to that of T cells in the defenses against virally infected cells and tumors. Thus, if downregulation of class I molecules results in an escape from T cell recognition, cells lacking class I molecules (or single alleles) become susceptible to NK cell lysis. In addition, preliminary experimental data *in vitro* suggest that NK cells may also play a role in rejection of bone marrow grafts in humans (115). NK cells, characterized by a limited repertoire and by their ability to destroy cells that lack given class I alleles, are thought to represent a first and rapid line of defense. The readiness of NK cell intervention reflects the fact that these cells constitutively express a lytic machinery and are thus ready to kill. In addition, in view

of their limited repertoire, the frequency of NK cells displaying a given specificity is relatively high. Thus, for NK cells, neither activation nor clonal expansion may be strictly required for a rapid elimination of potentially harmful cells.

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MHC Class I-Deficient Mice

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I. Introduction

Major histocompatibility complex (MHC)-encoded molecules play critical roles in the protective immune response, in various immunopathological reactions, and in reactions against transplanted tissues. The central function of class I MHC molecules appears to be the presentation on the cell surface of small peptide fragments of endogenously produced antigens for recognition by $CD8^+$ cytotoxic T lymphocytes (CTLs) (Yewdell and Bennink, 1992). Thus class I molecules present for T cell recognition foreign antigenic peptides derived from intracellular pathogens such as viruses, intracellular bacteria, and other organisms that replicate intracellularly. It is believed that the protective immune response against many of these agents depends critically on the destruction of pathogen-infected cells by CTLs, and the consequent limitation of pathogen replication. In some cases, however, an aberrant $CD8^+$ T cell response triggered by class I-associated antigens can lead to severe, even lethal, immunopathological reactions. The specific roles of class I and class II MHC molecules in various protective and immunopathological reactions have been subjected to intensive investigation, but much remains to be learned.

The development of the T cell repertoire is critically dependent on interactions with MHC molecules, which are exceedingly genetically polymorphic. Developing T cells must be rendered tolerant of self-MHC antigens and of autologous peptides presented by self-MHC molecules. Self-tolerance in the T cell compartments is accomplished in part by the clonal deletion, within the thymus, of nascent T cells with strong reactivity to self antigens (negative selection) (Blackman *et al.*, 1990).

In addition, successful development of T cells requires that they undergo positive selection by recognition of self-MHC molecules (von Boehmer, 1992). It is believed that positive selection serves to select for maturation those T cells that are able to interact preferentially with foreign antigenic peptides bound to self-MHC molecules. Because the foreign antigens are not usually present at the time of T cell matura-

tion, it has been suggested that low-affinity interactions between MHC molecules expressed by thymic cells and developing T cells suffice for positive selection. The positive selection process is believed also to determine the phenotype, $CD4^+$ or $CD8^+$, of developing T cells with receptors specific for class II MHC or class I MHC, respectively. In addition to their function in presenting foreign antigens for T cell recognition, recent studies indicate an important role for class I molecules in regulating the activity of natural killer (NK) cells. In the case of NK cells, normal expression of class I molecules by target cells inhibits target cell lysis by NK cells. Based on these observations, it has been proposed that one role of NK cells is in the elimination of cells in which class I expression is inhibited by pathogen defense mechanisms or random mutation (Ljunggren and Karre, 1990; Raulet, 1992).

Class I molecules are highly polymorphic, and is has long been appreciated that class I antigenic differences are a major barrier to successful organ or tissue transplantation. Even when a donor and host express identical class I molecules, the presentation by class I molecules of polymorphic minor histocompatibility antigens can lead to strong rejection reactions. Disparity at class II MHC genes can also lead to tissue rejection, however, and the relative contribution of class I and class II molecules to the histoincompatibility of different tissues continues to be a subject of extensive investigation, with important clinical implications.

Class I molecules are composed of a membrane-spanning heavy chain of approximately 45 kDa, and a light chain of approximately 12 kDa, β_2-microglobulin (β_2m). In mice, there are two to three distinct polymorphic MHC class I heavy genes, called *K*, *D*, and *L*. In addition, a large number of relatively nonpolymorphic class I molecules, called class Ib molecules, are encoded in the region telomeric to the MHC (Stroynowski, 1990). Several other genes encoding class I-like (β_2m-associated) molecules of unknown function, called CD1 molecules, are on a distinct chromosome (Stroynowski, 1990). The function of most class Ib molecules is unknown. In contrast to the many class I genes in an individual, β_2m is encoded by a single gene.

The class I heavy chain forms three extracellular domains, α1, α2, and α3. The amino-terminal α1 and α2 domains form the peptide-binding groove of the molecule, whereas the membrane-proximal α3 domain contains the binding site for CD8 (Bjorkman and Parham, 1990). β_2m is bound noncovalently to the class I heavy chain proximal to the α3 domain, and is not anchored in the membrane. β_2m can dissociate from class I heavy chains and a considerable level of free β_2m is found in serum (Bernabeu *et al.*, 1984).

Much remains to be learned concerning protective immunity, transplantation, lymphocyte development, and immunopathological reactions, and mice deficient for expression of MHC molecules obviously represent a powerful approach to address all of these areas. However, given the large number of class I genes in individual animals, mutants in individual class I genes are generally of limited utility. Studies with mutant cell lines deficient for expression β_2m have demonstrated that β_2m is important for normal cellular expression of class I molecules (Hyman and Stallings, 1976, 1977). In the absence of β_2m, the cell surface levels of all functional class I molecules were severely reduced. Hence mutation in the intact animal of a single gene, the gene for β_2m, was expected to result in a general class I deficiency. The realization of this possibility came with the advent of the gene-targeting methodology for generating mutations at defined gene loci by homologous recombination and the capacity to reconstitute the mouse germ line with totipotent embryonic stem cells (Capecchi, 1989). In the first successful application of the complete method, the β_2m gene was mutated in mice, yielding class I-deficient mice (Zijlstra *et al.*, 1989; Koller and Smithies, 1989). This review addresses the current status of studies of class I-deficient mice, including studies of T cell recognition, NK cell responses, lymphocyte development, transplantation, protective immune responses, and immunopathological reactions.

II. Production of β_2-Microglobulin-Deficient Mice

Mice with a disrupted β_2-microglobulin gene were produced in two laboratories, by a nearly identical strategy (Zijlstra *et al.*, 1989; Koller and Smithies, 1989). In both cases, a DNA fragment containing the neomycin resistance gene, driven by the herpes simplex virus thymidine kinase gene, was inserted into a genomic clone of the β_2m gene, at an *Eco*RI site in the second exon of the gene. These targeting vectors were transfected into embryonic stem (ES) cells by electroporation. After selection of G418-resistant colonies, homologous recombinants were identified by polymerase chain reaction and confirmed by Southern blotting. The frequency of homologous recombinants compared to G418-resistant recombinants was estimated as 1/117 to 1/25.

By introducing the targeted ES cell clones into blastocysts, chimeric animals capable of germ-line transmission of the disrupted β_2m gene were readily prepared in both laboratories. The $\beta_2m^{+/-}$ heterozygous progenies of the founder animals were interbred to generate homozygous $\beta_2m^{-/-}$ mice (Zijlstra *et al.*, 1990; Koller *et al.*, 1990). The ES cells

employed to produce both β_2m^- strains were of 129 strain origin. In some cases β_2m-deficient 129 inbred mice, generated by crossing the founder animals to 129 mice, were employed for subsequent experiments. However, mice from both laboratories were backcrossed several times to the C57BL/6J strain, and many of the subsequent studies of the mice have employed these backcrossed animals. The β_2m gene has also been backcrossed to H-2^k mice (Zijlstra *et al.*, 1992; Bix and Raulet, 1992a). Based on the nearly identical mutations in the two mutant strains, and the failure to detect β_2m protein in either strain, it is highly unlikely that they differ in their critical properties. For this reason, they will be considered interchangeable in the following discussion, except in instances where different experimental results might possibly be attributable to the use of the different strains. Though differences among the mutants remain possible, it is most likely that the different experimental results thus far observed with the two strains are due to differences in the specific experimental procedures rather than to differences in the β_2m mutations employed to generate the strains. For the sake of convenience, in the discussion that follows $\beta_2m^{-/-}$ mice will be referred to simply as β_2m^- mice, and $\beta_2m^{+/+}$ and $\beta_2m^{+/-}$ mice will be referred to as β_2m^+ mice. Although it was reported that the level of serologically detectable class I molecules was lower on cells from $\beta_2m^{+/-}$ than from $\beta_2m^{+/+}$ cells (Koller *et al.*, 1990), no significant biological differences between $\beta_2m^{+/-}$ and $\beta_2m^{+/+}$ mice have yet been reported.

III. β_2-Microglobulin-Deficient Mice Show No General Developmental Deficiencies

Based mostly on speculative considerations, but also on the early expression of β_2m in embryonic development, it had been suggested that class I molecules play important roles in cellular interactions during embryonic development of nonlymphoid tissues (Curtis and Rooney, 1979; Bartlett and Edidin, 1978). Other results suggested that class I molecules function as hormone receptors (Verland *et al.*, 1989; Kittur *et al.*, 1987; Hansen *et al.*, 1989; Schreiber *et al.*, 1984; Solano *et al.*, 1988). Obviously the β_2m^- animals represent a relatively stringent test of whether class I molecules play important developmental or physiological roles outside of the immune system. $\beta_2m^{+/-}$ heterozygous mice appeared to be normal. Among the offspring of the intercross of $\beta_2m^{+/-}$ heterozygous mice, $\beta_2m^{-/-}$ mice appeared at the expected 25% frequency, and no gross developmental defects in any organ systems were detectable (Zijlstra *et al.*, 1990; Koller *et al.*, 1990). When

housed under specific pathogen-free conditions, the mice survived to a normal old age and were fertile. These observations demonstrated that β_2m does not play an essential role in development of most organs. To the extent that class I molecules are dependent on β_2m for functional expression (see below), a similar conclusion is suggested for the role of class I molecules in embryonic development.

It has been suggested that β_2m plays an important role in the chemoattraction of hematopoietic stem cells to the thymus (Dargemont *et al.*, 1989). However, in the β_2m^- mice, the thymus contains normal numbers of developing T cells, even early in development, indicating that β_2m fails to play an essential role in migration of cells to the thymus (Zijlstra *et al.*, 1990; Koller *et al.*, 1990).

Finally, it has been demonstrated that a β_2m-associated protein functions as an IgG Fc receptor in neonatal gut cells (Simister and Mostov, 1989). In line with this observation, it was shown that neonatal gut cells from β_2m^- mice fail to bind IgG (Zijlstra *et al.*, 1990).

IV. Effects of β_2-Microglobulin Deficiency on Cell Surface Expression of Class I Molecules

Mice homozygous for the β_2m mutation are severely deficient in the cell surface expression of most if not all class I molecules. However, in the case of at least some class I molecules, a low level of class I molecules on the cell surface could be detected with serological and/or functional tests.

A. Serological Analysis

Using multiple monoclonal antibodies reactive with K^b, Qa-2, K^k, and D^k to stain lymphoid cells of $\beta_2m^{-/-}$ H-2^b or H-2^k mice, no detectable reactivity could be detected by flow cytometry (Zijlstra *et al.*, 1990; Koller *et al.*, 1990; Bix and Raulet, 1992a; L. Corral, M. Bix, and D. Raulet, unpublished data). However, specific staining of H-2^b β_2m^- lymphoid cells was detected with several monoclonal antibodies (mAbs) reactive to D^b, although the level of staining was reduced approximately 20-fold compared to the staining of wild-type cells (Bix and Raulet, 1992a; Zijlstra *et al.*, 1990). Surface expression of D^b on β_2m^- cells was not unexpected, since earlier studies showed that D^b can be detected on the surface of β_2m-mutant cell lines (Allen *et al.*, 1986). In the earlier studies, however, it was reported that only mAbs reactive to the D^b α3 domain, and not those reactive to the α1 or α2 domain, would stain β_2m-deficient cells, suggesting that the critical α1 and α2 domains were in an unfolded configuration (Allen *et al.*, 1986).

In contrast, lymphoid cells from β_2m mice stained at approximately the same relative level with α1-specific mAb as with α3-specific mAb (Bix and Raulet, 1992a; Zijlstra *et al.*, 1990). Binding of α1-specific mAb occurred even when no exogenous β_2m was present, i.e., when the staining was performed in serum-free medium, with purified mAb. Addition of human or bovine β_2m to the cells had little or no effect on surface expression levels of D^b. Reexamination of β_2m-mutant, D^b-transfected tumor cells showed that binding of α1-specific mAb could be detected on these cells also (Bix and Raulet, 1992a), in contrast to the findings of the original studies of these cells. These results suggested that the peptide and T cell receptor binding domains of the D^b molecule might be able to fold properly in the absence of β_2m, in contrast to earlier reports. However, the levels of these folded molecules on β_2m^- cells were very low.

B. Low Level of Functional Class I Molecules on β_2-Microglobulin-Deficient Cells

Earlier studies suggested that D^b molecules expressed on the surface of β_2m-deficient tumor cell lines are nonfunctional as target antigens for T cell recognition (Allen *et al.*, 1986), consistent with the notion that the α1/α2 domains of these molecules are folded improperly. In contrast to these earlier findings, it was shown that lymphoid blasts from β_2m^- mice serve as targets for class I allospecific CTLs (Bix and Raulet, 1992a; Glas *et al.*, 1992; Zijlstra *et al.*, 1990). CTLs specific for D^b class I molecules lysed β_2m^- targets, and the lysis was inhibited by anti-Db mAb (Bix and Raulet, 1992a). When the CTLs were specific for K^b, lysis was also observed, despite that fact that K^b molecules could not be reliably detected on the cell surface by serological methods (Bix and Raulet, 1992a; Glas *et al.*, 1992).

When the CTLs were generated by stimulation with β_2m^+ allogeneic cells, the cytotoxicity of β_2m^- targets was very weak, however, requiring 10–50 times more CTLs to achieve the same level of lysis as β_2m^+ targets (Bix and Raulet, 1992a; Glas *et al.*, 1992; Zijlstra *et al.*, 1990). When the CTLs were generated by stimulation with β_2m^- cells, the cytotoxicity of β_2m^- targets was similar to or even better than the lysis of β_2m^+ targets (Bix and Raulet, 1992a; Glas *et al.*, 1992).

Lysis of allogeneic β_2m^- target cells occurred equally in serum-free medium and serum-containing medium, suggesting that exogenous β_2m was not necessary for functional recognition of class I molecules on β_2m^- cells (Bix and Raulet, 1992a; Glas *et al.*, 1992). Lysis occurred even when the CTLs were mutant for β_2m (i.e., were derived from β_2m^- hosts), arguing that transfer of β_2m from the effector CTL to the

target cell was not necessary to observe lysis (Bix and Raulet, 1992a). These results suggest that functional class I molecules exist on the surface of β_2m^- cells and therefore that β_2m is not essential for functional activity of class I molecules in all cases. It should be emphasized, however, that functional class I expression is strongly diminished on β_2m^- cells in comparison with β_2m^+ cells.

The available data do not address whether some class I heavy chains exist on the cell surface as functional monomers that can be recognized by allospecific CTLs; i.e., unbound by β_2m and peptide. It appears more likely that in the absence of β_2m, some endogenous peptides bind class I heavy chains, resulting in the formation of properly conformed dimers of stability sufficient to reach the cell surface. Consistent with this possibility is the finding that short peptides can bind to free class I heavy chains in detergent lysates, resulting in the proper folding of the class I heavy chain in the absence of β_2m (Elliott *et al.*, 1991). If such peptide–class I dimers reach the cell surface, even transiently, they may be recognized by allospecific CTLs or by CTLs specific for minor histocompatibility antigens presented by class I molecules. Given the very low levels of class I molecules detected at the surface of β_2m^- cells, it is likely that such complexes would be of low stability and/or would represent only a subset of available endogenous peptides.

In support of the possibility that endogenous peptides can be presented on the class I molecules of β_2m^- cells, it was reported that H-2^b-restricted CTLs specific for minor histocompatibility antigens could lyse β_2m^- target cells presenting these minor H antigens (Glas *et al.*, 1992). However, it is not clear whether the two strains used to generate the minor H-specific CTLs, A.SW and B6, differ in their expression of class Ib molecules (Shen *et al.*, 1982). Thus, it was not excluded that the lysis of β_2m^- targets was mediated by CTLs specific for class Ib molecules. No lysis of β_2m^- male target cells by H-Y-specific CTLs was observed. Hence further studies are necessary to definitively establish whether β_2m^- cells can functionally present endogenously produced peptides.

V. Transplantation Studies

The creation of MHC-deficient mice prompted the suggestion that mutant animal tissues, devoid of MHC expression, could be used an universal donor tissue for transplantation to humans (Zijlstra *et al.*, 1990; Koller *et al.*, 1990). The success of such a strategy depends on whether such tissue would in fact fail to be rejected by the host im-

mune system. Following is a discussion of the studies published to date concerning transplantation of β_2m^- tissues to normal mice.

A. Hematopoietic Cell Grafts

In the case of hematopoietic cell transplants in mice, β_2m^- fetal liver and bone marrow cells were vigorously rejected by normal hosts, even those host animals that were otherwise genetically identical to the donor (Bix *et al.*, 1991). This rejection was mediated by NK cells (see Section VI, where this phenomenon is discussed in more detail). Hence, instead of facilitating transplantation of hematopoietic cells, class I deficiency represents a strong barrier to transplantation of these cells. Because previous studies suggested that susceptibility of tissues to rejection by NK cells is predominantly a property of hematopoietic cell transplants (Yu *et al.*, 1992), these results do not bear directly on the likelihood of success in transplanting nonhematopoietic tissues.

B. Skin Grafts

Several studies have been performed to assess whether β_2m-deficient skin grafts can be rejected by normal mice. Skin grafts from β_2m^- mice were rejected by both MHC-different hosts and by MHC-identical hosts that differed at multiple minor histocompatibility (minor H) loci (Zijlstra *et al.*, 1992; Markmann *et al.*, 1992). Little if any delay in rejection was observed with either type of graft. β_2m^- skin grafts transplanted to otherwise syngeneic β_2m^+ recipients were uniformly accepted, demonstrating the requirement for allogenicity for rejection and arguing against a role for NK cells in rejecting class I-deficient skin (Zijlstra *et al.*, 1992) (see Section VI).

Several explanations can be considered for the rejection of allogeneic β_2m^- skin: (1) class II molecules (or class II molecules complexed with minor H antigens) were targeted for rejection; (2) free β_2m in the recipient serum associated with free class I molecules on donor skin, resulting in increased levels of class I sufficient for rejection; (3) the low level of functional, β_2m-free class I molecules on β_2m^- cells served as target antigens for rejection.

Some of these possibilities were addressed in experiments with double-mutant mice that are deficient for expression of class I and class II molecules (Grusby *et al.*, 1993). Such animals were prepared by breeding the β_2m^- mutant mice with mice mutant for the class II $A\beta^b$ gene. The latter mice were generated by disrupting the $A\beta$ gene by homologous recombination in embryonic stem cells (Grusby *et al.*, 1991). It should be noted that H-2^b mice fail to express the $E\alpha$ gene and hence are negative for expression of class II E molecules. It was found

that skin grafts from $\beta_2m^-A\beta^-$ mice were rejected by MHC-different hosts with only slightly delayed kinetics compared to the rejection of wild-type grafts (Grusby *et al.*, 1993). This finding tends to argue against the possibility that class II molecules are responsible for rejection of β_2m^- skin grafts. On the other hand, it was reported that rejection of $\beta_2m^-A\beta^-$ skin grafts was delayed if $CD4^+$ T cells were depleted from the recipients, or if $A\beta^-$ mice (which are deficient in $CD4^+$ T cells) were used as recipients. These observations raise the possibility that the $A\beta^-$ cells continue to express class II molecules that can be targeted by $CD4^+$ T cells. However, class II molecules have not been detected on the $A\beta^-$ cells by any other detection methods. A second possibility is that antigens are shed from the skin graft, processed, and presented by antigen-presenting cells of the host (Auchincloss *et al.*, 1993); conceivably, a local chronic reaction against these antigens could lead to rejection even though the graft cells cannot themselves be recognized. A third possibility was suggested, i.e., that a novel mechanism of transplantation rejection, independent of the class I and class II MHC, is revealed in the double-mutant mice (Grusby *et al.*, 1993).

To address the possibility that the class I molecules on β_2m^- skin were reconstituted with host serum-derived β_2m, $\beta_2m^-A\beta^-$ skin was transplanted to β_2m^- recipients that differed at multiple minor *H* loci. As was seen with wild-type recipients, rapid rejection of the donor grafts occurred (Grusby *et al.*, 1993). These observations indicate that reconstituting donor class I molecules with β_2m from the host is not essential for graft rejection.

The possibility that class I heavy chains free of β_2m on β_2m^- skin can be targeted for rejection by $CD8^+$ T cells remains possible, although this was considered unlikely (Grusby *et al.*, 1993). In the case of $\beta_2m^-A\beta^-$ skin transplanted to β_2m^- recipients, the recipient is deficient in the production of $CD8^+$ T cells, further diminishing the likelihood that residual class I expression is responsible for rapid rejection of the β_2m^- grafts. But this deficiency is not complete, and as discussed in Section VII, B, class I-specific $CD8^+$ CTLs can be detected in some β_2m^- mice after they reject allogeneic β_2m^+ skin grafts. Since allospecific CTLs were able to lyse β_2m^- concanavalin A (Con A) blast targets *in vitro* (Section IV, B), such CTLs could potentially cause rejection of β_2m^- skin. It might be expected, however, that class I-specific rejection of β_2m^- skin would be delayed compared to the rejection of β_2m^+ skin.

Given the indication that $CD4^+$ T cells participate in rejection of $\beta_2m^-A\beta^-$ skin (Grusby *et al.*, 1993), and presumably also in the rejec-

tion of β_2m^- skin, it is difficult from the present data to dissect the role of class I-specific $CD8^+$ CTLs in the rejection of β_2m^- skin. The $CD4^+$ T cell response may obscure the $CD8^+$ T cell response to residual class I molecules on the graft. In order to resolve whether the class I molecules on β_2m^- skin grafts can serve as target antigens for graft rejection, it will be necessary to test rejection of β_2m^- skin that differs from the host solely in class I antigen expression, and/or to employ as graft recipients mice that are thoroughly depleted of $CD4^+$ T cells.

In contrast to the nearly normal rejection of β_2m^- skin differing at the MHC or at multiple minor H antigens, β_2m^- male skin was accepted by nearly all (9/11) β_2m^+ female recipients (Zijlstra *et al.*, 1992). Control β_2m^+ male skin grafts were rejected by each of 11 β_2m^+ females. Therefore, rejection based on recognition of the H-Y antigen requires β_2m expression by the engrafted skin cells. Hence, the success of transplantation of β_2m^- skin appears to depend on the specific antigenic disparity.

C. Pancreatic Islet Grafts

In contrast to β_2m^- skin grafts, the majority (4/7) of H-2^b β_2m^- pancreatic islet grafts survived indefinitely when transplanted to fully allogeneic H-2^d hosts, whereas all β_2m^+ islet grafts were rejected quite rapidly (Markmann *et al.*, 1992). These results indicate that class I deficiency is sufficient to prolong graft survival of at least some tissues. The observation that class I deficiency prolongs islet cell grafts, but not skin grafts, may reflect the low or absent class II expression by islet cells under normal circumstances. In the absence of class II expression, it would be expected that class I molecules would be required to initiate the antigraft response and serve as target molecules for rejection.

On the other hand, three of the seven β_2m^- islet grafts were rejected by allogeneic hosts. The mechanism of rejection was not established. One possibility is that class II molecules are variably induced on the engrafted cells, resulting in rejection by $CD4^+$ T cells. Targeting of the low levels of class I molecules on β_2m^- cells is another possibility.

The survival of β_2m^- islet cell grafts in allogeneic autoimmune nonobese diabetic (NOD) mice, a mouse model for autoimmune diabetes, was also investigated (Markmann *et al.*, 1992). In this case, all the β_2m^- islet grafts were rejected. These results concord with previous studies showing that $CD4^+$ T cells are the primary effector cells for autoimmune damage to transplanted islets in NOD mice (Wang *et al.*, 1991).

D. Liver Cell Grafts

Grafts of β_2m^- liver cells, transplanted under the kidney capsule of allogeneic or xenogeneic recipients, showed prolonged survival compared to β_2m^+ liver cells. Hence, the rejection of liver cell grafts, like islet cells, may depend primarily on class I molecules (Li and Faustman, 1993).

However, some of the recipients rejected the β_2m^- liver cell grafts, and there was evidence of rejection in most of the recipients. In an attempt to address whether NK cells might be contributing to rejection of class I-deficient cells, 129 strain β_2m^- liver cells were transplanted to wild-type 129 strain mice. Although donor liver cells survived for at least 30 days in all the recipients, there was evidence for a mild rejection reaction, based on a lymphocyte infiltrate and the presence of dead and dying liver cells in the graft sites (Li and Faustman, 1993). Although this was attributed to NK cells, which had been previously shown to attack syngeneic class I-deficient hematopoietic cells, there remains the possibility that the recipient and donor differed in their expression of minor H antigens: the 129 substrain used to generate the β_2m^- mutant (129terSv) (described in Li *et al.*, 1992) differs from the 129 substrain (129/J) employed as recipients in these experiments. Hence, further experiments will be necessary to demonstrate a role for NK cells in liver cell rejection.

Regardless of whether NK cells participate in rejection of β_2m^- liver cells, the rejection of β_2m^- liver cell grafts by fully allogeneic recipients was stronger than the rejection attributed to NK cells (Li and Faustman, 1993). Therefore, it is likely that other effector mechanisms, such as $CD4^+$ T cells, or $CD8^+$ T cell recognition of residual class I molecules on β_2m^- liver cells, participate in the relatively mild rejection of β_2m^- liver cell grafts.

E. Summary of Transplantation Results

The available data suggest that transplantation of class I-deficient tissue to allogeneic recipients is problematic. In part because of the possibility of $CD4^+$ T cell reactions against class II molecules on these grafts, it has been thus far difficult to dissect the success with which the β_2m mutation prevents class I-mediated rejection reactions. Further experiments will be necessary to address this important question. Nevertheless, success in transplanting some β_2m^- tissues, including pancreatic islet cells and liver cells, suggests that transplantation strategies exploiting MHC-deficient grafts may be feasible.

VI. Effects of Class I Deficiency on Target Cell Susceptibility to NK Cells

Studies of the susceptibility of tumor cells to lysis by natural killer cells led to the observation that susceptibility to lysis is correlated with lower levels of cell surface expression of class I molecules on target cells (Karre *et al.*, 1986; Ohlen *et al.*, 1989; Ljunggren *et al.*, 1990; Shimuzu and DeMars, 1989; Sturmhöfel and Hämmerling, 1990). These observations provoked the suggestion that class I MHC expression protects target cells from lysis by NK cells.

Corroboration of this effect came from analysis of the destruction of cells from β_2m^- mice by NK cells. The first studies showed that transplants of fetal liver or bone marrow cells from β_2m^- mice were rejected by irradiated normal mice of the same (or different) MHC genotype (Bix *et al.*, 1991). Even large doses of fetal liver cells (3×10^7) were rejected, resulting in the death of the recipients from hematopoietic failure. Rejection was prevented if NK cells were depleted from the recipients by pretreating them with anti-NK1.1 antibodies.

Subsequent studies demonstrated that NK cells from normal mice lyse β_2m^- T cell blasts *in vitro* (Liao *et al.*, 1991; Hoglund *et al.*, 1991). B cell blasts from β_2m^- mice are also lysed by NK cells (M. Bix, L. Corral, and D. Raulet, unpublished data). The lysis of β_2m^- cells may be restricted to hematopoietic cell types. NK cells failed to lyse cells of an embryonic fibroblast line established from β_2m^- embryos (Zijlstra *et al.*, 1992). Furthermore, there is little indication that grafts of nonhematopoietic tissues from β_2m^- mice to normal mice are rejected by NK cells (see Section V).

Thus, NK cells recognize and destroy otherwise normal hematopoietic cells that fail to express class I molecules. The results are important for interpreting earlier observations that H-2 heterozygous (F_1 hybrid) animals often reject bone marrow grafts from H-2 homozygous parental strains (hybrid resistance) (Cudkowicz and Stimpfling, 1964; Yu *et al.*, 1992). Although other interpretations have been proffered as explanations for hybrid resistance, the results with β_2m^- transplants fit best with the "missing self hypothesis," which proposes that NK cells reject cells that fail to express the full complement of self class I molecules (Ljunggren and Karre, 1990). Hence the rejection of parental cells by F_1 hybrids is likely due to the failure of the graft to express the other parental MHC haplotype.

More recent studies suggest that a principal mechanism for rejection of class I-deficient cells is that NK cells express inhibitory receptors for specific target cell class I molecules; engagement of these receptors by MHC molecules on target cells prevents lysis by NK cells (Karlhofer *et*

al., 1992; Raulet, 1992). The receptors are related members of a small family of molecules encoded in the NK gene complex, of which Ly49 is the best characterized example (Yokoyama *et al.*, 1990; Karlhofer *et al.*, 1992). Because β_2m^- cells fail to engage these receptors and thus fail to inhibit the NK cell lytic machinery, β_2m^- cells are destroyed by NK cells.

VII. Deficiencies in Lymphocyte Development in β_2-Microglobulin Mice

Not unexpectedly, class I-deficient mice manifest a profound deficiency in the development of mature $CD8^+$ T cells. Less expected is a deficiency in the development of $TCR\alpha\beta^+CD4^-CD8^-$ T cell subset. The development of a subset of class I-dependent $\gamma\delta$ T cells may also be impaired in β_2m^- mice. Finally, NK cell activity is abnormal in these mice. Each of these deficiencies will be discussed below.

A. Normal Development of $CD4^+$ T Cells and B Cells?

The question remains whether the development of other mature lymphocyte subsets, that is, $CD4^+$ T cells and B cells , is normal in the class I-deficient mice. $CD4^+$ T cells and B cells are found in normal numbers in β_2m^- mice (Zijlstra *et al.*, 1990; Koller *et al.*, 1990). $CD4^+$ T cell function as determined in mixed lymphocyte reactions was normal, and various studies have demonstrated cytokine production by these cells in β_2m^- mice infected with various pathogens (see Section IX). The repertoire of $V\beta$ regions expressed by $CD4^+$ T cells in β_2m^- mice was quite similar to the pattern observed in control β_2m^+ mice (N. Liao, M. Bix, and D. Raulet, unpublished data). Considerable B cell function (and $CD4^+$ T helper cell function) in β_2m^- mice can also be inferred from the substantial antibody responses observed in responses to some antigens (Lehmann-Grube *et al.*, 1993; Fiette *et al.*, 1993; Rajan *et al.*, 1992; Spriggs *et al.*, 1992).

However, there are also indications that the function of the $CD4^+$ and/or B cell compartments may not be completely normal in β_2m^- mice. Total serum levels of several IgG isotypes were reportedly significantly reduced in nonimmune β_2m^- mice (Spriggs *et al.*, 1992), and in some antigen responses IgG antibody production was considerably depressed (Spriggs *et al.*, 1992), whereas in others it was substantial though delayed (Lehmann-Grube *et al.*, 1993). These instances will be discussed again below in the review of the responses of β_2m^- mice to various infectious agents (see Section IX). The extent and cause(s) of the alterations in the antibody response remain unclear. In the case of the immune response to infectious agents, it is possible that the ab-

sence of the $CD8^+$ T cell response influences the antibody response indirectly by altering the extent, course, or site of infection. More interestingly, $CD8^+$ T cells may play a previously unappreciated role in influencing the quality of the $CD4^+$ T cell response to an antigen, for example, by biasing the response toward Th1 or Th2 type, which would then affect the type of class switching that occurs (Swain *et al.*, 1991). Alternatively, the cytokines produced by $CD8^+$ T cells may act directly on B cells to promote or inhibit class switching. Further studies will be necessary to resolve these questions.

B. $TCR\alpha\beta^+CD8^+CD4^-$ T Cell Development

1. Deficient Development of $TCR\alpha\beta^+CD8^+$ T Cells

a. $CD8^+$ T Cell Development in Thymus and Lymphoid Organs

$TCR\alpha\beta^+CD8^+CD4^-$ T cells recognize antigens bound to class I molecules, and many previous studies demonstrated that successful development of mature $CD8^+$ T cells requires positive selection on class I molecules expressed in the thymus (von Boehmer, 1990). Indeed, the profoundly depressed expression of class I molecules in β_2m^- mice leads to a dramatic deficiency in the development of $CD8^+$ T cells (Zijlstra *et al.*, 1990; Koller *et al.*, 1990; Bix and Raulet, 1992b). Only 1–5% of the normal complement of $TCR\alpha\beta^+CD8^+CD4^-$ cells can be detected in the thymus or periphery of β_2m^- mice. Accordingly, spleen cells from β_2m^- mice fail to mount detectable cytotoxicity against allogeneic target cells after primary sensitization *in vitro* (Zijlstra *et al.*, 1990; Bix and Raulet, 1992b). These results substantiate the importance of interactions of developing T cells with class I molecules in the subsequent differentiation of $CD8^+$ T cells.

b. Residual $CD8^+$ T Cell Function in β_2-Microglobulin-Deficient Mice

Although the deficiency in $TCR\alpha\beta^+CD8^+$ CTL precursors in β_2m^- mice is profound, it does not appear to be absolute. It was shown that β_2m^- mice reject allogeneic skin with normal kinetics; though this rejection may be attributable at least in part to the activity of class II MHC-specific $CD4^+$ T cells (Section V,B), $CD8^+$ CTL precursors specific for allogeneic class I molecules were detectable in the spleens of some (but not all) recipients following rejection of the graft (Zijlstra *et al.*, 1992). More striking results were obtained by inoculating β_2m^- mice with allogeneic class I^+ tumor cells. The tumors were rejected, and following rejection strong alloclass I-specific $CD8^+$ CTL activity could be detected in the host peritoneal exudates (Apasov and Sitkovsky, 1993).

The appearance of $CD8^+$ T cells in β_2m^- mice occurred even in hosts that had been thymectomized prior to inoculation of tumor cells (M. Zijlstra and R. Jaenisch, unpublished data). Therefore, it is unlikely that the appearance of $CD8^+$ T cells in these mice is due to migration to the thymus of released β_2m from the skin graft or tumor cells. Rather, it appears likely that a small number of $CD8^+$ T cells develop in the β_2m^- mice, and that allospecific precursors can be specifically amplified from this population following strong immunization, as with a skin graft or growing tumor cells. The origin of the functional $CD8^+$ T cells in β_2m^- mice is unknown. They may arise from thymic cells that are positively selected by low levels of class I molecules expressed on thymic cells (see Section IV), or they may represent a small set of $CD8^+$ T cells that differentiates independently of class I recognition.

The existence of small numbers of $CD8^+$ T cells in β_2m^- mice emphasizes the necessity for caution in interpreting studies in which, for example, β_2m^- mice resist infections (see Section IX), suggesting that $CD8^+$ T cells do not play an essential role in resistance to the infection. But it should also be emphasized that for the $CD8^+$ T cells in β_2m^- mice to function normally in resisting an infectious agent, class I antigen presentation must also occur, and this is clearly impaired in β_2m^- mice. Nevertheless, given the suggestive evidence that some presentation of peptides may occur in the absence of β_2m (see Section IV), definitive demonstration that $CD8^+$ T cells do not play a role in a process requires that other methods, such as depletion of $CD8^+$ T cells with antibody treatments *in vivo*, be combined with the use of β_2m^- mice (e.g., see Section IX).

c. *Development of $TCR\alpha\beta^+CD8^+$ T Cells in Intestinal Epithelium*

The β_2m mutation also has a profound effect on the development of $TCR\alpha\beta^+CD8^+$ T cells among intestinal intraepithelial lymphocytes (i-IELs). This is of interest because in normal mice T cells among i-IELs differ from T cells in lymphoid organs in several respects (Rocha *et al.*, 1992). Many i-IELs develop in athymic mice, leading to the hypothesis that a substantial fraction of i-IELs normally develop extrathymically, presumably in the gut (Guy-Grand *et al.*, 1991; Klein, 1986). About a third of the i-IELs in normal mice are $TCR\alpha\beta^+$ T cells, and most of these (80–90%) are $CD8^+$. Unlike peripheral $CD8^+$ T cells in lymphoid organs, however, 50–75% of $TCR\alpha\beta^+CD8^+$ i-IELs fail to express the $CD8\beta$ chain, and hence express only $CD8\alpha$ homodimers at the cell surface (Guy-Grand *et al.*, 1991). It has been suggested that

failure to express the CD8β chain is a property of CD8$^+$ T cells that mature extrathymically. Interestingly, β_2m$^-$ mice manifested a severe (>95%) deficiency in TCR$\alpha\beta^+$CD8α^+ i-IELs (Correa *et al.*, 1992). Because most of these cells in wild-type mice are CD8β^-, it can be inferred that both the CD8α/α^+ and CD8α/β^+ subsets of TCR$\alpha\beta^+$ i-IELs are dependent on class I for normal development. Therefore, although many of these cells may be thymus independent, they are nonetheless class I dependent for development. In contrast, the TCR$\gamma\delta^+$CD8$^+$ T cells among i-IELs were present in normal numbers in β_2m$^-$ mice (Correa *et al.*, 1992) (see Section VII,D); many of these cells in normal mice also fail to express the CD8β chain and are thought to develop extrathymically (Goodman and Lefrançois, 1988). Therefore, the $\gamma\delta^+$ i-IELs are apparently not class I dependent for their development.

2. *CD8$^+$ T Cell Development Studied with β_2m$^-$ Mice*

a. Thymic Cell Types Mediating Positive Selection

Many studies have argued for a preeminent role for thymic epithelial cells in inducing positive selection of CD4$^+$ and CD8$^+$ T cells. Studies with chimeric mice and with MHC transgenes whose expression was directed to different cell types have suggested that MHC expression by radioresistant thymic epithelial cells is essential for positive selection (Lo and Sprent, 1986; Kisielow *et al.*, 1988; Marrack *et al.*, 1988; Benoist and Mathis, 1989; Blackman *et al.*, 1989; Bill and Palmer, 1989). The idea that thymic epithelial cells are uniquely able to direct positive selection has influenced mechanistic models proposed to explain the process. For example, it was proposed that thymic epithelial cells present a unique set of peptides, bound to MHC molecules, that are necessary to achieve sufficient binding of T cell receptors for positive selection to proceed (Marrack *et al.*, 1989).

The cell types directing positive selection of CD8$^+$ T cells were examined with the use of β_2m$^-$ mice and hematopoietic chimeras. In irradiated β_2m$^+$ mice repopulated with β_2m$^-$ fetal liver, the hematopoietic cells within the thymus are mostly β_2m$^-$ cells, whereas the thymic epithelial cells are β_2m$^+$ cells. It was found that high levels of CD8$^+$ T cells differentiated under these conditions, consistent with the expectation that thymic epithelial cells efficiently direct positive selection (Bix and Raulet, 1992b; Hoglund *et al.*, 1991).

In irradiated β_2m$^-$ mice repopulated with β_2m$^+$ fetal liver, the hematopoietic cells within the thymus are mostly β_2m$^+$ cells whereas the thymic epithelial cells are β_2m$^-$ cells. Contrary to the expectation that CD8$^+$ T cells would not differentiate under these circumstances, a

significant number of mature $CD8^+$ T cells, about one-sixth the number found in normal mice, developed in the thymus and populated the periphery (Bix and Raulet, 1992b). These cells expressed the $CD8\alpha$ and $CD8\beta$ chains, they were of mature phenotype (i.e., negative for expression of heat-stable antigen), and they were functional by the criteria that allospecific and antigen-specific CTLs could be generated from the population. The differentiation of these cells depended on β_2m expression by hematopoietic cells, because a comparable population was not observed in chimeras in which β_2m^- fetal liver was used to reconstitute irradiated β_2m^- mice.

There was a possibility that β_2m shed from donor hematopoietic cells was transferred to recipient thymic epithelial cells, increasing class I expression by these cells to a level sufficient to direct positive selection. Such effects of β_2m have not been observed *in vitro*, i.e., the lysis of β_2m^- blasts by allospecific CTLs was equal whether or not β_2m was added to the medium (Bix and Raulet, 1992a; Glas *et al.*, 1992) (Section IV,B); reconstitution of functional class I expression of β_2m^- cells appears to require the addition of relatively high concentrations of optimally sized class I-binding peptides, in addition to β_2m (Hogquist *et al.*, 1993). Nevertheless, the possibility of β_2m transfer *in vivo* was addressed experimentally. Chimeras were produced in which the MHC antigens of the donor and recipient differed: $H\text{-}2^{b/k}$ heterozygous β_2m^+ fetal liver cells were used to reconstitute irradiated $H\text{-}2^b$ β_2m^- recipients. It was expected that if the $CD8^+$ T cells were positively selected by hematopoietic cells in the chimeras, the restriction specificity of antigen-specific CTLs from these animals would be for $H\text{-}2^b$ or $H\text{-}2^k$ (donor type), whereas if reconstituted thymic epithelial cells were responsible for selection only $H\text{-}2^b$-restricted CTLs would be detected. The results showed approximately equal representation of minor H-specific CTLs restricted by $H\text{-}2^k$ and $H\text{-}2^b$, providing strong evidence that selection was mediated by hematopoietic cells in these chimeras (Bix and Raulet, 1992b). In contrast, when β_2m^+ $H\text{-}2^b$ recipients were repopulated with $H\text{-}2^{b/k}$ β_2m^+ fetal liver cells, the CTLs were predominantly restricted by $H\text{-}2^b$, as expected. The results indicate that when thymic epithelial cells are class I deficient, hematopoietic cells can direct positive selection.

These data indicate that hematopoietic cells can direct positive selection of $CD8^+$ T cells, albeit inefficiently, and argue against a unique role of thymic epithelial cells in presenting peptides essential for positive selection. In line with this, other data raise the possibility that the peptides involved in positive selection may be expressed on many cells, not just thymic epithelial cells (Hogquist *et al.*, 1993). The exist-

ing data do not refute the hypothesis that signaling of immature thymocytes by thymic epithelial cells is essential for differentiation of $CD8^+$ T cells, because even in the β_2m^- thymus, developing T cells are expected to develop in close juxtaposition to thymic epithelial cells. The data do suggest, however, that developing T cells need not recognize the MHC molecules actually expressed by thymic epithelial cells. Interestingly, a similar analysis of $CD4^+$ T cell differentiation in chimeras in which class II-deficient hosts are repopulated with class II^+ hematopoietic stem cells resulted in the conclusion that $CD4^+$ T cell differentiation requires that thymic epithelial cells express class II molecules (Markowitz *et al.*, 1993). The reasons for this difference between the differentiation of $CD4^+$ T cells and $CD8^+$ T cells are currently unclear. One possibility is that class I but not class II molecules are expressed on a sufficiently large number of hematopoietic cells in the thymic cortex to direct positive selection.

b. Selective versus Instructive Models of T Cell Development

A controversial area of current research concerns the mechanism by which developing T cells differentiate to become $CD4^+$ T cells or $CD8^+$ T cells, depending on the specificity of their T cell receptors for class II or class I MHC, respectively (von Boehmer and Kisielow, 1993). The "instructive" model suggests that the interaction of $CD4^+CD8^+$ T cells with class II and class I MHC "instructs" the cell to extinguish expression of CD8 or CD4, respectively. The "stochastic/selection" model, in contrast, suggests that $CD4^+CD8^+$ T cells randomly extinguish CD8 or CD4 expression; successful differentiation then requires selection of the cells by class II or class I MHC, respectively. Only cells whose T cell receptor specificity matches the MHC specificity of the randomly retained CD8 or CD4 coreceptor will be successfully selected. Previous studies attempted to test the stochastic/selection model by the combined use of T cell receptor and CD8 transgenes. These experiments failed to yield positive evidence for the stochastic selection hypothesis, and thus tended to support the instructive model of T cell differentiation (Robey *et al.*, 1991; Borgulya *et al.*, 1991).

The stochastic/selection hypothesis suggests that there should exist immature CD4 or CD8 "single-positive" intermediates in T cell development. Furthermore, since these populations arise stochastically, they should exist in mice deficient for class I or class II MHC expression. Evidence has been obtained for a relatively small $CD4^+CD8^-$ population in class II-deficient mice (Chan *et al.*, 1993). These cells have an unusual cell surface phenotype, and may be immature. Conversely, it was reported that class I-deficient mice contain a small

population of TCR$\alpha\beta^+$CD8$^+$CD4$^-$ cells within the thymus. This population was minor and not well characterized, however, and it remains unclear whether it differs from the small number of CD8$^+$ T cells, which are apparently functionally mature, that were previously detected by flow cytometric studies and in functional experiments (see Section VII,B,1).

Interestingly, in mice that were both class II deficient and β_2m deficient, the development of the CD4$^+$CD8$^-$ "intermediate" population was abrogated (Chan *et al.*, 1993). These results were the basis for a model of T cell differentiation in which the engagement of the TCR by class I *or* class II MHC molecules triggers stochastic differentiation to CD4$^+$ or CD8$^+$ "single-positive" phenotype. The model proposes that the final maturation of these intermediate cells would require selection by MHC molecules, which necessitates matching of the TCR specificity with the coreceptor specificity (Chan *et al.*, 1993).

The stochastic selection model has also been addressed more directly with the use of β_2m$^-$ mutant mice expressing a CD4 transgene (Davis *et al.*, 1993). The stochastic/selection model accounts for the failure of β_2m$^-$ mice to develop substantial numbers of mature CD8$^+$ cells by suggesting that cells that randomly extinguish CD4 expression require class I engagement for subsequent selection. The differentiation of these cells should be rescued if all developing T cells are induced to express CD4. When the β_2m$^-$ mice were crossed with a mouse harboring a CD4 transgene driven by the *lck* promoter, which is expressed at very high levels in the thymus, substantial numbers of CD8$^+$ T cells of mature phenotype were found in the thymus and periphery. The experiment was repeated with a CD4 transgene driven by the CD3δ gene promoter, which was expressed in the thymus at levels similar to normal CD4 expression levels. In this case, the extent of differentiation of CD8$^+$ T cells was considerably less substantial but still evident. These results suggest that CD4 coreceptor expression can rescue the development of CD8$^+$ T cells in class I-deficient mice, and thus lend support to the stochastic/selection model. However, the inefficiency of the rescue observed with normal levels of CD4 transgene expression has not been accounted for, nor have the earlier results with TCR/CD8 double-transgenic mice. The final resolution of the difficult problem of lineage determination in T cell development will require additional experimentation.

c. Peptide–MHC Complexes Involved in Positive Selection

A central question in understanding the formation of the T cell repertoire is whether positive selection of developing T cells is mediated by recognition of peptides in the groove of MHC molecules, and

the nature and specificity of such peptides. Mice harboring MHC class I molecules with defined mutations in residues in the peptide-binding groove have been employed in previous studies to suggest that alterations in peptide binding alter the specificity of positive selection (Nikolic-Zugic and Bevan, 1990; Sha *et al.*, 1990). However, the nature of the peptides that mediate positive selection and whether they represent a subset of MHC-binding peptides was not established in these studies.

With the use of β_2m-deficient mice, a thymic organ culture system has been developed in which the capacity of specific peptides to mediate positive selection can be determined (Hogquist *et al.*, 1993). The system exploits the aforementioned observation that class I heavy chains can reach the cell surface in the absence of β_2m, as well as the finding that functional class I–peptide complexes can be formed from free class I heavy chains incubated with sufficient concentrations of β_2m and appropriate specific class I-binding peptides. In the case of β_2m^- spleen cells from H-2^b mice, it was shown that K^b-specific presentation of an ovalbumin peptide ($OVA_{257-264}$) occurred in the presence of relatively high concentrations of peptide and β_2m (Hogquist *et al.*, 1993).

It was shown that organ cultures of thymi from β_2m^- mice failed to develop significant numbers of $CD8^+$ T cells unless peptides and β_2m were supplied in the culture medium. In the initial experiment, the organ cultures were provided at initiation with β_2m and two peptides, one presented by K^b ($OVA_{257-264}$) and the other by D^b (influenza $NP_{366-374}$). Compared to control organ cultures without peptides, a small but significant increase (approximately threefold) in the yield of $CD8^+$ T cells was observed after 10 days of organ culture (Hogquist *et al.*, 1993).

Reasoning that the extent of positive selection might be limited by the frequency of precursor T cells that can bind these specific peptides, more complex mixtures of peptides expected to bind K^b were tested. These mixtures were synthesized from degenerate oligonucleotide templates in *Escherichia coli* and contained octamer peptides in which four of the residues were fixed, including the "anchor" residues for K^b binding. Peptide mixtures of increasing complexity were obtained by preparing extracts of pools of increasing numbers of bacterial colonies and purifying the synthetic peptide mixture. The peptide mixtures, added at the initiation of thymic organ culture, resulted in substantial increases in the production of $CD8^+$ T cells, and the more complex peptide mixtures resulted in higher yields of $CD8^+$ T cells (Hogquist *et al.*, 1993). It was also observed that heterogeneous prepa-

rations of natural peptides extracted from spleen cells were able to stimulate the production of substantial numbers of $CD8^+$ T cells.

Taken together, the results from this organ culture system suggest that various class I-binding peptides, including antigenic peptides and natural "self" peptides derived from nonthymic cells (spleen cells), are able to direct positive selection. Because self peptides derived from nonthymic cells appear able to drive positive selection, the results argue against models in which specific thymic peptides are necessary to direct positive selection (see also Section VII,B,2,a). This system should be quite useful for determining the properties of peptides that direct positive selection, particularly if the system is combined with the use of mice whose T cells all express the same TCR specificity, i.e., TCR transgenic mice.

C. Development of $TCR\alpha\beta^+CD4^-CD8^-$ T Cells

Although the large majority of mature T cells express CD4 or CD8, a small subset of $TCR\alpha\beta^+$ T cells in the thymus and in the peripheral lymphoid organs is characterized by its lack of expression of CD4 or CD8 (Budd *et al.*, 1987; Fowlkes *et al.*, 1987; Ceredig *et al.*, 1987). Most of these cells are mature by several criteria: their expression of phenotypic markers is characteristic of mature cells (i.e., $CD5^{hi}$, $Qa\text{-}2^+$, and HSA^-) and they secrete cytokines (e.g., IL-4) and proliferate in response to stimulation with anti-TCR complex monoclonal antibodies. The biological function of these cells has not been determined, but several possible functions have been proposed, including roles in immune reactions to mycobacterial antigens (Porcelli *et al.*, 1992), tumor immunity (Levitsky *et al.*, 1991), and immune suppression (Palathumpat *et al.*, 1992). Human T cell lines of this phenotype, derived from peripheral blood by stimulation with mycobacterial antigens, have been shown to interact with CD1 molecules (Porcelli *et al.*, 1992). CD1 molecules are similar to class I molecules in their domain organization and in their association with β_2m. However, they are only distantly related to other class I molecules at the amino acid sequence level and they are encoded by genes on a chromosome distinct from that encoding the MHC (Stroynowski, 1990). It was proposed that $TCR\alpha\beta^+CD4^-CD8^-$ cells are specialized for recognition of microbial antigens presented by CD1 or perhaps other class I-like molecules such as the class Ib molecules (Porcelli *et al.*, 1992; Bix *et al.*, 1993).

An interesting property of $TCR\alpha\beta^+CD4^-CD8^-$ cells in the mouse is their frequent utilization of a single family of $V\beta$ regions in their TCR. Approximately 50–70% of $TCR\alpha\beta^+CD4^-CD8^-$ cells in most mouse

strains express one of the three Vβ8 family members, and about two-thirds of those express a single Vβ8 family member, Vβ8.2 (Budd *et al.*, 1987; Fowlkes *et al.*, 1987; Ceredig *et al.*, 1987). These results suggested that this population is subject to strong selection. However, significant differences in Vβ usage were not observed in comparisons of mice of different MHC genotypes (Takagaki *et al.*, 1989; Budd *et al.*,1987; Takahama *et al.*, 1991). Furthermore, in mice expressing TCRαβ$^+$ transgenes, relatively large numbers of transgene-expressing CD4$^-$CD8$^-$ cells appeared regardless of whether the mice expressed the MHC molecule known to be necessary for selection of transgene-expressing CD8$^+$ T cells. Based on these findings it was suggested that this population is not subject to positive selection (von Boehmer *et al.*, 1991; von Boehmer, 1992).

A different picture emerged when this population was examined in β_2m$^-$ mice (Bix *et al.*, 1993). The size of the Vβ8$^+$ subset of TCRαβ$^+$CD4$^-$CD8$^-$ thymocytes was decreased by approximately 80% in β_2m$^-$ mice. The size of the Vβ8.2 subset was decreased by 90%. The TCRαβ$^+$CD4$^-$CD8$^-$ population as a whole was decreased by only 50%, suggesting that most Vβ8$^+$CD4$^-$CD8$^-$ T cells, but not most Vβ8$^-$TCRαβ$^+$CD4$^-$CD8$^-$ cells, are dependent on selection by class I molecules. The failure to observe evidence for positive selection of TCR transgenic (Vβ8.2$^+$) CD4$^-$CD8$^-$ T cells may be a consequence of the inappropriate expression of the transgenes in TCRγδ$^+$ T cells, most of which do not require class I expression for positive selection (von Boehmer, 1992).

Although these data indicate that the Vβ8$^+$CD4$^-$CD8$^-$ population in normal mice is subject to a form of positive selection by class I molecules, studies of the development of this population in hematopoietic chimeras suggest that the mechanism of selection differs significantly from the mechanism of selection of conventional CD4$^+$ or CD8$^+$ T cells. Efficient selection of conventional CD4$^+$ and CD8$^+$ T cells requires expression of class I molecules by radioresistant thymic cells, presumably thymic epithelial cells, whereas class I expression by hematopoietic cells is not essential. In contrast, normal development of Vβ8$^+$CD4$^-$CD8$^-$ thymocytes does not require class I expression by thymic epithelial cells, whereas class I expression by hematopoietic cells is essential (Bix *et al.*, 1993). These data suggest that the selection of Vβ8$^+$CD4$^-$CD8$^-$ cells by class I molecules involves interactions with hematopoietic cells rather than thymic epithelial cells. The different rules controlling differentiation of TCRαβ$^+$CD4$^-$CD8$^-$ T cells compared to conventional T cells suggests a distinct role for these cells in the immune system. Analysis of

the β_2m^- mice, which are deficient for these cells, may help determine their function *in vivo*.

D. Effects of Class I Deficiency on Development of $\gamma\delta^+$ T Cells

The specificity of TCR$\gamma\delta$ T cells for antigens and antigen-presenting molecules is poorly understood (Raulet, 1989; Brenner *et al.*, 1988). Several TCR$\gamma\delta^+$ T cell lines reactive with conventional class I and class Ib molecules have been isolated, raising the possibility that class I molecules serve as antigen-presenting molecules for $\gamma\delta$T cells (Matis *et al.*, 1987; Bluestone *et al.*, 1988; Porcelli *et al.*, 1989; Bonneville *et al.*, 1989). Because the antigens recognized by $\gamma\delta$ cells are in most cases not known, it has not been possible so far to use cells from the β_2m^- mice to test the general role of class I molecules as presenting molecules for $\gamma\delta$ T cells. However, it has been possible to ask whether the recognition of class I molecules is necessary during the developmental phase of $\gamma\delta$ cells, in analogy to the requirement for class I recognition for the development of $CD8^+$ T cells. In fact, no deficiency was detected in the β_2m^- mice in the development of $\gamma\delta$ cells in the thymus, secondary lymphoid organs, and intraepithelial locations (Correa *et al.*, 1992; Zijlstra *et al.*, 1990; Koller *et al.*, 1990). The usage of Vγ and Vδ regions was normal, and the cells were functional as measured by proliferation induced by anti-TCR$\gamma\delta$ antibodies. If anything, there was a slight increase in the frequency and reactivity of $\gamma\delta$ cells in various compartments in the β_2m^- mice compared to normal mice. These results led to the conclusion that most $\gamma\delta$ cells do not require positive selection on class I MHC molecules. A similar conclusion may be reached regarding the role of class II MHC in the development of $\gamma\delta^+$ T cells, based on the report that normal numbers of splenic $\gamma\delta^+$ T cells are found in double-mutant mice deficient for both class I and class II MHC (Grusby *et al.*, 1993).

The apparent normality of $\gamma\delta$ T cells in β_2m^- mice extended to TCR$\gamma\delta^+CD8^+$ T cells in lymphoid organs and among intestinal intraepithelial lymphocytes (Correa *et al.*, 1992). TCR$\gamma\delta^+$ T cells that express CD8 might be expected to be the most likely candidates for class I-dependent TCR$\gamma\delta^+$ T cells. $CD8^+$ cells represent a minor fraction of $\gamma\delta^+$ T cells in lymphoid organs (less than 10%), but a substantial fraction (70-90%) of $\gamma\delta^+$ T cells found in the intestinal intraepithelial lymphocyte population (Goodman and Lefrançois, 1988; Bonneville *et al.*, 1988). The failure to find deficiencies in these TCR$\gamma\delta^+CD8^+$ populations suggests these cells develop independently of class I expres-

sion. In contrast TCR$\alpha\beta^+$CD8$^+$ i-IELs were profoundly diminished in β_2m$^-$ mice (see Section VII,B,1).

In two other studies, the possible role of class I molecules in the development of specific TCR$\gamma\delta$ was examined. The results obtained suggested an important role for class I in the development of these specific $\gamma\delta^+$ T cells (Wells *et al.*, 1991; Pereira *et al.*, 1992). Both studies employed TCR$\gamma\delta$ genes from cell lines known to be specific for class I molecules, specifically class Ib molecules encoded in the Tla region. The rearranged γ and δ genes from these cell lines were used to produce transgenic mice in which most of the T cells express the transgenic TCR. These transgenic mice were bred with the β_2m$^-$ mice to produce class I-deficient transgenic animals. In both studies, the development of the transgene TCR$^+$ T cells was profoundly inhibited if the mice were also class I deficient (Wells *et al.*, 1991; Pereira *et al.*, 1992). In the thymus most of the transgene TCR$^+$ cells expressed an immature HSA$^+$ phenotype. In the periphery the number of cells expressing the transgenic TCR was considerably reduced in the β_2m$^-$ mice compared to β_2m$^+$ controls.

These results suggested that the development of a subset of $\gamma\delta$ cells requires positive selection by interactions with class I molecules. However, most $\gamma\delta$ cells appear to develop independently of MHC expression. The question remains whether undefined non-MHC presenting molecules exist for the majority of $\gamma\delta$ cells. Selection by such molecules may be important for development of most $\gamma\delta^+$ T cells. Alternatively, development of many $\gamma\delta$ T cells may not require selection.

VIII. Impaired NK Cell Activity in Class I-Deficient Mice

As discussed in detail earlier, bone marrow stem cells, lymphoblasts, and possibly other cell types from β_2m$^-$ mice are susceptible to destruction by NK cells from normal mice (Section VI). If the NK cells from β_2m$^-$ mice mediated similar activity, it would be expected that autologous tissues would be attacked. In fact, the class I-deficient mice show no generalized hematopoietic effects that might be expected to result from autoreactive NK cells. Furthermore, grafts of fetal liver cells from β_2m$^-$ mice were not rejected by irradiated β_2m$^-$ mice, but were rejected by control irradiated normal mice (Bix *et al.*, 1991). *In vitro* studies demonstrated that enriched populations of NK cells from poly(I:C)-induced β_2m$^-$ mice failed to lyse β_2m$^-$ Con A blast targets (Liao *et al.*, 1991; Hoglund *et al.*, 1991). The deficiency in NK activity in class I-deficient mice was also evident in assays of rejection of

allogeneic cells: normal irradiated H-2^b mice reject allogeneic (H-2^d) bone marrow cells in a reaction mediated by NK cells, but β_2m-deficient H-2^b mice fail to reject H-2^d bone marrow cells (Liao *et al.*, 1991). The spleens of β_2m^- mice contained normal numbers of NK1.1^+ cells (Liao *et al.*, 1991), suggesting that the deficit is in the reactivity of the NK cells and does not reflect the deletion or impaired production of NK cells. Taken together, these results suggest a generalized impairment in class I-deficient mice of several activities mediated by NK cells.

The full extent of NK deficiency in β_2m^- mice is still to be resolved. NK cells from β_2m^- mice retained some ability to lyse the standard NK tumor target, YAC-1 (Liao *et al.*, 1991; Hoglund *et al.*, 1991), although the lysis of these cells was usually reduced compared to lysis by wild-type NK cells (Liao *et al.*, 1991). In some studies, pathogen-infected β_2m^- mice developed NK activity, as detected by lysis of YAC-1 target cells (Hou *et al.*, 1992). This may reflect the existence of a component of NK activity, specific for YAC-1 and possibly some other tumor target cells, which is not affected by the class I-deficient environment. It is not known whether the residual NK activity in β_2m^- mice is protective against pathogens. Therefore, in considering the possible effector cells responsible for protective immune responses mediated by β_2m^- mice, it cannot be assumed that the NK response is completely deficient.

Aside from the deficiency of NK cells in β_2m^- mice, other indirect evidence suggests that the class I MHC environment influences the reactivity of NK cells. The most obvious evidence is the fact that NK cells, as measured in assays of bone marrow rejection, seem to develop "self-tolerance." For example, though NK cells in F_1 mice reject parental or H-2-allogeneic bone marrow grafts, they do not reject autologous bone marrow grafts (Bennett, 1987). The development of tolerance in the NK cell compartment is poorly understood, and the β_2m-deficient mice should be useful tools to begin to develop an understanding of the process.

IX. Responses of Class I-Deficient Mice to Pathogens

The deficiency in class I expression would be expected to affect immune responsiveness in several ways. Aside from the defect in class I presentation per se, β_2m^- mice are severely deficient in $CD8^+$ T cells. Furthermore, they are impaired in NK cell activity, deficient in the complement of TCR$\alpha\beta^+CD4^-CD8^-$ cells, and may be deficient in a subset of $\gamma\delta$ cells that recognizes class I molecules. Given the defects

in cells other than $CD8^+$ T cells in the β_2m^- mice, it should be emphasized that susceptibility of class I-deficient mice to a pathogen does not necessarily mean that $CD8^+$ T cells are essential mediators of immunity to the pathogen. Nevertheless, the β_2m^- mice represent an excellent system to test the role of class I molecules in protective immune responses.

A. Viral Infections

One of the earliest activities ascribed to $CD8^+$ CTLs was the lysis of virus-infected cells (Doherty and Zinkernagel, 1974; Zinkernagel and Doherty, 1979). Indeed, it has been generally assumed that $CD8^+$ T cells play a critical role in immunity to most viral infections. The results of several previous studies involving selective depletion of $CD8^+$ T cells *in vivo*, or adoptive transfers of purified $CD8^+$ T cells, have supported the hypothesis that they play a significant role in viral clearance (Ada and Jones, 1986; Kast *et al.*, 1986). The creation of class I-deficient mice has allowed this important question to be reexamined by an independent method.

1. Influenza Virus

Although influenza virus is not a natural mouse pathogen, mice infected intranasally with the virus develop a temporary viral pneumonia. Athymic mice develop a persistent infection that usually leads to death, establishing a role for T cells in resistance to infection (Wells *et al.*, 1983).

β_2m^- mice, like β_2m^+ control animals, were able to clear the A/HKx31 (H3N2) influenza A virus by 10–13 days after intranasal infection (Eichelberger *et al.*, 1991). Similar results were obtained when $CD8^+$ T cells were depleted by antibody treatments of normal mice. Depletion of $CD4^+$ T cells (by treating mice with anti-CD4 antibody) also caused little delay in viral clearance. Because nude mice or mice depleted of both $CD4^+$ and $CD8^+$ T cells die after infection with this virus (Eichelberger *et al.*, 1991), the results suggest that either $CD8^+$ or $CD4^+$ T cells can independently accomplish clearance of this influenza virus. The mechanism by which $CD4^+$ T cells clear influenza virus is not known, but several possibilities can be considered, including their role in helping antibody responses and/or direct cytotoxicity of infected cells by $CD4^+$ CTLs. Evidence was presented that the β_2m^- mice generate low levels of $CD4^+$, class II-restricted CTL activity *in vitro*. The results of secondary adoptive transfer experiments suggest, however, that in normal mice $CD8^+$ T cells play the major role in clearing influenza virus (Ada and Jones, 1986).

Although the aforementioned experiments suggest that under some conditions of infection normal class I expression is not essential for clearing influenza virus, they do not address whether class I expression accelerates viral clearance or is critical under stringent conditions of infection. In a separate study (Bender *et al.*, 1992), it was shown that the A/Port Chalmers/1/73 (H3N2) influenza A virus is cleared by β_2m^+ but not β_2m^- mice at 8 days postinfection. β_2m^- mice eventually cleared this infection, by day 15 postinfection. Because this virus, like the HKx31 virus, is of relatively low virulence, the delayed kinetics raised the possibility that the β_2m^- mice would be highly susceptible to a more virulent influenza virus. In fact, infection with the virulent A/Puerto Rico/8/34 influenza virus resulted in substantial (90%) mortality of β_2m^- mice by 8–10 days postinfection, but only 20–30% mortality of β_2m^+ animals (Bender *et al.*, 1992). The authors conclude that the excess mortality was due to the deficiency of $CD8^+$ T cells, although this issue was not directly addressed. Taken together, the results for influenza virus suggest that under conditions of mild infection or infection with an influenza virus of low virulence, class I presentation is dispensable for immune protection, but under conditions of a more serious infection, class I expression is indispensable.

2. *Vaccinia Virus*

The poxvirus, vaccinia virus, generally leads to a mild temporary infection in normal mice, but leads to a generalized, lethal infection in athymic mice (Spriggs *et al.*, 1992). β_2m^- mice infected intradermally in the tail with various doses of vaccinia virus exhibited patterns of lesions similar to those in β_2m^+ controls, and the lesions healed with similar kinetics (Spriggs *et al.*, 1992). Because vaccinia virus is not typically lethal for mice unless administered intracranially, the results are reminiscent of the previously mentioned studies demonstrating resistance of β_2m^- mice to relatively nonvirulent influenza viruses. Furthermore, the intradermal tail route of immunization did not lead to virus spread in any of the animals studied, somewhat limited the conclusions that can be drawn from this study concerning the role of $CD8^+$T cells in generalized vaccinia virus infections.

Class I molecules and $CD8^+$ T cells are not known to play a significant direct role in promoting antibody responses. It was therefore a surprising observation that the secondary IgG antibody response to vaccinia virus infection was substantially weaker in β_2m^- mice than in β_2m^+ control mice; in contrast, the titers of vaccinia-specific IgM and IgE antibodies were normal (Spriggs *et al.*, 1992). The levels of total serum IgG isotypes in β_2m^- mice were also reduced significantly

compared to controls (three- to eight-fold, depending on the isotype), even before immunization. Total serum IgE and IgM levels were normal.The finding that the β_2m mutation does not affect the IgE response, which is dependent on T helper cells, suggests that the T helper response is not generally deficient in these mice. Moreover, antiviral IgG antibody responses of β_2m^- mice to lymphocytic choriomeningitis virus infection were strong though somewhat delayed compared to the responses of normal controls (see later). Further studies will be necessary to elucidate the generality and the cause of the observed deficit in IgG production. One possibility is that the β_2m^- mice have altered cytokine production due to the absence of $CD8^+$ T cells. $CD8^+$ T cells are known to produce substantial levels of interferon-γ (IFN-γ), which has been implicated in regulating class switching. It should be emphasized, however, that the deficit in IgG production affects all IgG isotypes tested, whereas IFN-γ is implicated in regulating the production of specific isotypes. The impaired development of some $TCR\alpha\beta^+CD4^-CD8^-$ T cells, of NK activity, and of a subset of $\gamma\delta$ cells could also have indirect effects on antibody production.

3. *Sendai Virus*

The murine parainfluenza type 1 virus, Sendai, is a major respiratory pathogen in mice. The results of various studies suggest that class I MHC-restricted CTLs play a critical role in clearing Sendai virus (Anderson *et al.*, 1980; Kast *et al.*, 1986). Indeed, when β_2m^- mice were infected with Sendai virus, they showed significantly delayed clearance of the virus and an increased mortality compared to β_2m^+ mice (Hou *et al.*, 1992). Nevertheless, most of the β_2m^- mice survived the infection, even when possible residual $CD8^+$ T cells in the animals were depleted with anti-CD8 antibody treatment. In contrast, all of the mice in which both $CD4^+$ and $CD8^+$ T cells were depleted by antibody treatment succumbed to the Sendai infection, indicating an important role for $CD4^+$ T cells in the absence of $CD8^+$ T cells. $CD4^+$ CTL activity restricted by class II molecules was detected in β_2m^- mice but not in wild-type mice. This is reminiscent of the response of β_2m^- mice to LCMV and suggests that a compensatory $CD4^+$ CTL response occurs in the absence of $CD8^+$ T cell responses. It has not been resolved whether the $CD4^+$ CTL response is indeed protective.

The results with β_2m^- mice contrast with earlier studies in which higher doses of Sendai virus were employed and which indicated a critical role for $CD8^+$ T cells (Kast *et al.*, 1986). Further studies will be necessary to test whether high doses of Sendai virus will be lethal for

the β_2m^- mice. If so, it would fit with the emerging picture that $CD8^+$ T cells play a necessary role under conditions of serious infection with several viruses.

4. *Theiler's Virus*

Theiler's virus is a murine picornavirus that infects the central nervous system (Brahic *et al.*, 1981). The infection induces a chronic demyelinating disease in susceptible strains that is reminiscent of the lesions observed in humans with multiple sclerosis. It has been hypothesized that the lesions result from immunopathological reactions mediated by $CD8^+$ T cells, but consistent evidence on this point is lacking. The role of $CD8^+$ T cells in clearing the virus in resistant strains was also unclear. Although class I-restricted T cell responses have not generally been considered essential for resistance to picornavirus infections, previous studies indicated a critical role of genes in the *H-2D* (class I) region for resistance to Theiler's virus infection (Rodriguez and David, 1985; Bureau *et al.*, 1992).

Mice of the $H\text{-}2^b$ haplotype are normally resistant to Theiler's virus infection. Most β_2m^+ $H\text{-}2^b$ mice were devoid of detectable viral antigens by 25 days postinfection. In contrast, viral antigens were detectable in almost all $H\text{-}2^b$ β_2m^- mice by 52 days postinfection (Fiette *et al.*, 1993). Despite the persistent infection, however, the β_2m^- mice did not succumb to early infection. In contrast, most athymic nude mice die within 3 weeks of infection with Theiler's virus. It was suggested that the inability of β_2m^- mice to clear the virus was due to the defective $CD8^+$ T cell response. A deficient NK response was deemed unlikely to be the cause, because nude mice are highly susceptible to Theiler's virus infection despite an enhanced NK response. β_2m^+ and β_2m^- mice developed similar titers of neutralizing antiviral antibodies, suggesting that the antibody response was intact in β_2m^- mice (Fiette *et al.*, 1993). The antibody response may explain the enhanced survival of infected β_2m^- mice compared to infected nude mice.

With respect to the demyelinating disorder, it was found that the β_2m deficiency failed to prevent demyelination. These results suggest that $CD8^+$ T cells may not be the cause of demyelination, as was previously thought (Fiette *et al.*, 1993).

5. *Lymphocytic Choriomeningitis Virus*

Intracerebral infection with lymphocytic choriomeningitis virus (LCMV), an arenavirus, leads to acute, usually fatal, neurological disease (Zinkernagel and Doherty, 1979). Previous studies established

that the disease pathology involves cell-mediated immune reactions and suggested that $CD8^+$ T cells exclusively mediated the disease (Moskophidis *et al.*, 1987). Analysis of the β_2m^- mouse has recently allowed the reinvestigation of the etiology of this acute disease, which represents the classical model of an immune cell-mediated immunopathology accompanying viral infection.

Infection of normal mice with LCMV by intravenous or intraperitoneal routes leads to less severe clinical symptoms than does intracerebral infection, and the virus is eventually cleared. In this system the role of class I molecules in clearing LCMV has been also recently examined with the use of the β_2m^- mice.

a. Immunopathology of LCMV

Intracerebral infection of β_2m^- mice led to a less severe but still significant disease compared to infection of β_2m^+ control animals (Muller *et al.*, 1992; Doherty *et al.*, 1993; Quinn *et al.*, 1993). The disease in β_2m^- mice was characterized by meningitis, wasting, and some mortality, although the severity of the disease varied in different studies as did the extent of mortality, which ranged from 0 to 75%. The variation may be due to differences in the background genes of the β_2m^- mice employed in the different studies or to differences in the viral isolate used.

Despite these differences, all of the studies agree that the β_2m^- mice exhibit a form of the immune-mediated disease, contrary to expectations. Furthermore, direct evidence was presented that the disease is mediated by $CD4^+$ T cells rather than by the small number of residual $CD8^+$ T cells in β_2m^- mice: depletion of $CD4^+$ T cells from β_2m^- mice before or shortly after infection abrogated symptoms, whereas depletion of $CD8^+$ T cells had no effect (Doherty *et al.*, 1993; Quinn *et al.*, 1993). Also, the disease was successfully adoptively transferred to irradiated infected β_2m^- recipients using immune splenocytes from β_2m^- mice, and it was shown that depletion of $CD4^+$ T cells but not $CD8^+$ T cells from this population abolished transfer of the disease. Finally, transfer of $CD8^+$-depleted spleen cells from normal β_2m^+ mice to irradiated β_2m^- mice also transferred the disease, suggesting that the $CD4^+$ T cells from β_2m^- mice are not unique in their ability to cause disease (Doherty *et al.*, 1993; Quinn *et al.*, 1993). Taken together, these results suggest that in the absence of $CD8^+$ T cells, $CD4^+$ T cells mediate a somewhat milder form of LCM disease.

The mechanisms by which $CD4^+$ T cells cause disease is uncertain. Class II MHC-restricted and LCMV-specific $CD4^+$ CTLs were detected in β_2m^- mice in two studies (Muller *et al.*, 1992; Doherty *et al.*,

1993), but not in another study (Lehmann-Grube *et al.*, 1993). In the latter case, the disease was less severe than in the studies where $CD4^+$ CTLs were detected, raising the possibility that the CD4 CTL activity mediates the disease. Although there are relatively few class II-positive cells that could serve as targets for $CD4^+$ CTLs in normal brain tissue, it was reported that intracerebral infection led to the induction of increased class II MHC expression in normal and β_2m^- brains (Muller *et al.*, 1992). Other possibilities are that the disease results from the effects of cytokines (e.g., TNFα) produced by the $CD4^+$ T cells or that the effect of $CD4^+$ T cells is mediated through the induction of antibody production.

The demonstration of $CD4^+$ class II-restricted CTLs in LCMV-infected mice is notable, because such cells are not detected in immune wild-type mice. These findings suggest that in the absence of the possibility of a $CD8^+$ CTL responses, $CD4^+$ cytolytic activity may arise as a compensatory mechanism.

b. LCMV Clearance

Although intracerebral infection of normal mice with LCMV leads to a fatal disease, intravenous infection leads to a milder disease and the virus is eventually cleared (Doherty *et al.*, 1990). In contrast, despite the absence of symptoms, β_2m^- animals infected intravenously with LCMV maintained high titers of the virus for at least 100 days after intravenous infection (Lehmann-Grube *et al.*, 1993).

The finding that class I expression plays a critical role in clearing the virus seems to contradict the conclusions of another study that investigated the capacity of mice harboring a mutant CD8α gene to clear LCMV (Fung-Leung *et al.*, 1991). The absence of CD8 leads to a profoundly diminished class I- restricted CTL response, as also observed in β_2m^- mice. Surprisingly, however, LCMV (the same strain used in the studies with β_2m^- mice) was efficiently cleared by $CD8^-$ mice. These findings led the authors to suggest that $CD4^+$ T cells can clear LCMV in the absence of $CD8^+$ T cells. If correct, this would imply that the $CD4^+$ T cells in β_2m^- mice are for some reason defective in their capacity to clear the virus. Consistent with this possibility is that the $CD8^-$ mice, but not the β_2m^- mice, mediate local delayed-type hypersensitivity (DTH) reactions to virus (Lehmann-Grube *et al.*, 1993). Earlier studies suggested that DTH reactions to LCMV are mediated in part by $CD4^+$ T cells. However, it cannot be excluded that class I molecules play a role in initiating the DTH response. The IgG antibody response, which normally depends on $CD4^+$ T cells, was delayed but reached substantial levels in β_2m^- mice. If the $CD4^+$ T

cell response is indeed deficient, the reasons for this are unclear (see Section VII,A).

There are several other ways to reconcile these data, aside from trivial explanations such as genetic background differences between the strains employed. First, because $CD8^-$ mice express normal levels of class I molecules, it is possible that $CD8^-$ class I-restricted T cells are responsible for clearing the virus. For example, although it appears that most T cells of the CD8 lineage fail to develop in $CD8^+$ mice, it is possible that a small number of class I-restricted T cells develop in this lineage. These cells cannot, of course, express CD8, but they may nonetheless mediate class I-restricted reactions sufficiently well to clear the virus (Lehmann-Grube *et al.*, 1993). Because class I-restricted CTLs were not detected in $CD8^-$ mice, it must be supposed that they mediate viral clearance via distinct effector mechanisms. Alternatively, it is possible that in $CD8^-$ mice class I-restricted T cells of the $TCR\alpha\beta^+CD4^+CD8^-$, $TCR\gamma\delta^+CD4^-CD8^-$, or $TCR\alpha\beta^+CD4^-CD8^-$ lineages mediate viral clearance. Evidence that subsets of the latter populations are diminished in β_2m^- mice is discussed in Section VII. Finally, it is possible that natural killer cells play a role in clearing LCMV in the $CD8^-$ mice. Although there is evidence that NK cells do not play an essential role in clearing LCMV in normal animals, they may become more important in the absence of $CD8^+$ T cells. As discussed in Section VIII, NK activity is partially impaired in β_2m^- mice. On the other hand, significant NK function, as detected by lysis of YAC-1 tumor cells, was detected in mice infected with LCMV (J. Frelinger, personal communication).

In conclusion, the findings suggest that class I expression is essential for clearing LCMV, although the explanation for this effect is not clear. The simplest idea, that $CD8^+$ T cells play a necessary role, seems to be contraindicated by the findings that $CD8^-$ mice clear LCMV. Further studies will be necessary to establish whether this reflects the activity of residual effector cells of the CD8 lineage, or alternatively points to an important role for distinct class I-dependent effector cells in LCMV clearance.

B. Intracellular Bacteria: *Mycobacterium tuberculosis*

Class I-restricted T cells are known to attack cells harboring intracellular bacteria and are believed to play an important role in immunity to these pathogens. In the case of *Mycobacterium tuberculosis*, although cellular immunity rather than humoral immunity is essential for resistance to infection (Hahn and Kaufmann, 1981; Orme and Collins,

1983), the relative roles of CD4 and CD8 T cells in controlling infection have been controversial. This issue has been reexamined with the use of β_2m^- mice.

Strikingly, 100% of β_2m^- mice died by 10 weeks after infection with *M. tuberculosis*, whereas all the wild-type controls survived (Flynn *et al.*, 1992). The differential survival correlated with differential bacterial loads at 6 to 8 weeks postinfection. In contrast, the avirulent *Mycobacterium bovis* vaccine strain, BCG, failed to cause a lethal infection in β_2m^- mice, correlating with clearance of the bacteria. Prior immunization with BCG slightly prolonged the survival of β_2m^- mice subsequently challenged with *M. tuberculosis*, but all of the animals eventually died. These results indicate that control of the virulent *M. tuberculosis* infection requires the activity of class I MHC molecules.

As in other cases in which the β_2m deficiency abrogated immunity to a pathogen, the deficiency in $CD8^+$ T cells was considered the most likely direct cause (Flynn *et al.*, 1992). However, the possible contributory roles of other class I-specific T cells or NK cells were not ruled out. Previous studies of mycobacterial immunity indicated an important role of the cytokines IFN-γ and TNFα, which activate macrophages to kill ingested mycobacteria. It is therefore noteworthy that only relatively small differences were observed in the levels of these cytokines produced by *in vitro*-stimulated spleen cells from infected β_2m^+ or β_2m^- mice (Flynn *et al.*, 1992). Although further studies will be required to definitively establish that the deficiency in $CD8^+$ T cells is responsible for increased susceptibility to *M. tuberculosis*, the finding of class I dependency in this protective immune response represents an excellent example of the utility of the β_2m-deficient mouse for studies of protective immune responses.

C. *Trypanosoma cruzi* Infection

Trypanosoma cruzi infection causes Chagas' disease, a severe, often lethal syndrome that afflicts millions of people in Latin America. The syndrome is complex, involving severe inflammatory reactions reminiscent of autoimmune reactions. In fact, there has been considerable debate over whether the disease pathology results from autoimmunity secondary to *T. cruzi* infection (Parham, 1992).

In mice, infection with *T. cruzi* is characterized by an acute and chronic tissue inflammation, but normal mice fail to develop parasitemia and typically survive infection. $CD4^+$ T cells, $CD8^+$ T cells, antibody production, and NK cells have been implicated in the protec-

tive immune response in earlier studies, but their relative roles have not been well established (Trischmann, 1983; Andrade, 1983; Araujo, 1989; Rottenberg *et al.*, 1988).

β_2m^- mice infected with *T. cruzi* developed parasitemia in the acute phase of the disease and died by 30 to 35 days postinfection, whereas control animals failed to develop parasitemia and survived (Tarleton *et al.*, 1992). The infected β_2m^- mice also failed to develop the characteristic acute-phase inflammatory response in muscle, which was previously suggested to be mediated by $CD4^+$ T cells. At death, the infected β_2m^- mice showed extremely high parasite loads but only a moderate inflammatory response, suggesting that death resulted from direct effects of the parasites. The production of IL-2 and IFN-γ by infected β_2m^- mice was, if anything, increased compared to normal mice, suggesting that the absence of a protective immune response was not due primarily to deficiencies in cytokine production. These results suggest that class I molecules play an important role in resistance to *T. cruzi* infection. Mice depleted of $CD8^+$ T cells with anti-CD8 antibody were similarly susceptible to *T. cruzi* infection (Tarleton, 1990), suggesting that the susceptibility of β_2m^- mice to *T. cruzi* is due to a deficiency in these cells. As in the other cases discussed, the potential roles of class I-dependent NK cells or $\gamma\delta$ T cells in this syndrome are still unclear.

D. *Leishmania major*

In humans, *Leishmania* species often cause a progressive, ultimately fatal infection. In mice, infection with *Leishmania major* mimics human infection with *Leishmania donovani*. *Leishmania major* protozoans are obligate intracellular parasites that replicate in macrophages. The progression of *L. major* infection in mice is complex and genetically controlled (reviewed in Locksley and Louis, 1992). An essential role for $CD4^+$ T cells in controlling the infection has been determined with the use of cell transfer experiments and mice mutant for MHC class II expression. The susceptibility of some inbred mouse strains to progressive infection is associated with a bias toward a Th2 type $CD4^+$ T cell response to infection; mice that make predominantly Th1 type responses to *L. major* resist infection. In addition to the role for $CD4^+$ T cells, cell depletion experiments suggest that $CD8^+$ T cells also play a role: mice depleted of $CD8^+$ T cells had enhanced development of lesions and decreased ability to clear the parasites. However the CD8-depleted mice eventually resisted the *L. major* infection.

The significance of the class I-restricted response to *L. major* has been investigated with the use of β_2m-deficient mice on a 129 strain

genetic background (Wang *et al.*, 1993). The 129 mice are normally resistant to *Leishmania*. It was found that β_2m^- mice, like the β_2m^+ control animals, resisted *Leishmania* infection. They failed to develop *Leishmania* lesions and no parasites could be cultured from tissues 8 weeks after infection. No $CD8^+$ T cells could be detected in the recovered β_2m^- mice. However, the possibility that residual $CD8^+$ T cells contributed to recovery was not rigorously excluded. Although these results suggest that $CD8^+$ T cells do not play a major role in resistance of mice to *L. major* infection, it remains possible that they accelerate parasite clearance, and may even be essential for resistance under more severe conditions of infection.

E. Human Filarial Parasites

The nematode parasite *Brugia malayi* is an agent of human lymphatic filariasis, a major tropical disease. Normal mice resist infection with *B. malayi*, but immunodeficient scid/scid mice are permissive for infection. β_2m^- mice were found to resist infection, suggesting that class I-restricted T cells are not essential for protective immunity (Rajan *et al.*, 1992). The possibility that clearance of the parasites was delayed in β_2m^- mice was not investigated, nor was the possibility that β_2m^- mice may fail to resist more severe infections.

X. Autoimmune Disease

Little work has been done concerning the effect of the β_2m mutation on autoimmune disease. In one study, however, it was reported that older β_2m^- mice develop a mild autoimmune diabetes (Faustman *et al.*, 1991). This evidence was part of a larger argument that class I deficiency is a significant cause of autoimmune diabetes in mice and humans. However, the evidence generated considerable controversy (Gaskins *et al.*, 1992; Wicker *et al.*, 1992), and other investigators found that β_2m^- mice failed to develop diabetes (M. Zijlstra and R. Jaenisch, unpublished data). Further evidence will be necessary to establish that β_2m^- mice are prone to the development of autoimmune diabetes.

XI. Summary and Conclusions

A great deal has already been learned from the analysis of β_2m-mutant mice, but it is clear that a great deal remains to be learned. A significant (though unanticipated) problem with this model system is that it is functionally leaky: residual functional class I expression can be detected in β_2m^- mice, and small numbers of functional $CD8^+$

lymphocytes are present in the animals. In many cases, this has frustrated the initial attempts at obtaining immediate definitive resolution of important questions regarding the function of class I molecules. This has occurred primarily in instances in which the class I-deficient mice fail to express an expected phenotype—for example, in studies showing that β_2m^- mice make adequate protective immune responses against certain intracellular pathogens, and are able to reject some allogeneic tissues with a relatively normal pace. On the other hand, it appears that combining the use of β_2m^- mice with other methods (for example, antibody-mediated depletion of $CD8^+$ T cells) is usually adequate to circumvent these difficulties. It remains to be seen whether other better class I deficiencies can be engineered—for example, large deletions of class I genes or mutations in transcription factors essential for class I gene expression.

The extent of immunocompetence of β_2m^- mice was somewhat surprising. It was widely expected that class I-deficient mice would be exquisitely sensitive to many viral infections, though the results indicate that sensitivity varies dramatically with the virus and conditions of infection. However, it appears that in lieu of one major arm of the immune system, compensatory immune mechanisms are in many cases able to deal with infection. Similar conclusions are developing from the analysis of several other recently generated mutant mice. Nevertheless, the results indicate a very important role for class I-directed responses in clearing infections mediated by various viral and parasitic agents, particularly in the case of more severe conditions of infection.

Although the class I-deficient mice were initially considered primarily a vehicle for analysis of the role of $CD8^+$ T cells, evidence is accumulating that they manifest deficiencies in several other types of lymphocytes, including NK cells, $TCR\alpha\beta^+CD4^-CD8^-$ cells, and a subset of $TCR\gamma\delta^+$ cells. This has been a boon for analysis of the development of these cells, but at the same time it has created difficulties in assigning a biological effect of the mutation to a specific lymphocyte deficiency. Furthermore, the responsible population cannot in most cases be identified by infusing purified lymphocyte populations from normal mice back into the β_2m^- mice: because essentially all of the cells in the β_2m^- mouse are deficient in the expression of target class I molecules, reinfused effector cells will be unable to react with the tissue. Resolution of this problem will likely come from the exploitation of other recently developed mutant models as well as novel mutant animals.

Despite these experimental shortcomings, however, MHC-deficient

mice have already proved to be a very powerful tool in the study of several important areas of immunobiology. It is becoming increasingly clear that a major new route of investigation of the immune system will involve the analysis of mice with various combinations of defined mutations. The present stage represents only the beginning.

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The Immune System of Mice Lacking Conventional MHC Class II Molecules

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The major histocompatibility complex has held a fascination for immunologists since its discovery over 30 years ago. Thus it is not surprising that loci within the MHC were among the first targets once it became possible to direct mutations into the genome via homologous recombination in embryonic stem cells (for review and references, see Ref. *1*). Here we will concentrate on the immune system of mice lacking the conventional MHC class II molecules.

Class II $\alpha:\beta$ dimers are known to be central regulators of the immune response, implicated in antigen presentation, T–B cell collaboration, and thymic education (for review and references, see Ref. 2). Although most immunologists acknowledge the role of class II $\alpha:\beta$ dimers in these diverse processes, they still argue—sometimes with passion—over the precise mechanisms involved. In addition, there has been some discussion over possible roles in other phenomena inside and outside the immune system: an influence on class I-restricted T cell responses (*3*), on the selection of the $\gamma:\delta$ T cell repertoire (*4–7*), on the differentiation of B cells (*8, 9*), and on the maturation and/or targeting of sperm (*10*). To provide a new approach for resolving some of these controversies, three groups have produced mice with directed mutations in MHC class II loci; these mice are referred to as II^0 mice (*11–13*).

There are four murine class II genes of known function—Aα, Aβ, Eα, and Eβ—and their products are considered to be the conventional class II molecules. In addition, there are the Oα/Oβ gene pair, whose products have been detected on a very limited subset of cells but have not yet been shown to act as restriction elements for T cells (*14, 15*), and the set of H-2M genes, which are known to be transcribed but have not yet been reported to give rise to proteins and, in fact, show as much homology to MHC class I as to class II molecules (*16*). Because it would be an extremely difficult technical feat to eliminate all of the class II genes and no others, all three groups aimed at abolishing expression of the conventional class II molecules. To avoid E expres-

sion, all began with embryonic stem cells derived form H-2^b haplotype mice, which do not display E molecules because of a deletion in the Eα promoter (*17*). To abrogate A expression, two groups (*11, 12*) mutated the Aβ gene and the third group (*13*) engineered an Aα mutation. The former approach has the advantage that one can subsequently introduce Eα transgenes and thereby produce E-only mice (discussed in Section III,A). The latter has the advantage of eliminating the theoretical possibility of mixed-isotype Aα : Eβ dimers, even if such molecules have never been found in b-haplotype mice. In fact, although rigorously sought (*11*), A_α^b : E_β^b dimers were never found in the Aβ knockouts and all three of the mutant strains exhibit the same phenotype according to numerous parameters.

A variety of criteria were used to establish that the II^0 mice are indeed devoid of conventional MHC class II molecules—cytofluorimetric (*11–13*), immunohistological (*11*), biochemical (*11*), and functional (*11–13*). When bred under specific-pathogen-free (SPF) conditions, these animals thrive. However, if housed in a conventional facility, they can display a subnormal growth rate, health status, and breeding performance. No aberrations have so far been reported for any organ system other than the immune system.

I. Peripheral Immune Compartments

A. $CD4^+$ T Cells

The most striking anomaly in II^0 mice, if perhaps the most expected, is the near-complete lack of $CD4^+$ T cells in the periphery. This is illustrated for the lymph node in Fig. 1, but is also true for the spleen, peritoneum, and bone marrow (S. Cardell, unpublished). A major functional consequence of this anomaly is the complete inability to make T-dependent antibody responses (discussed in Section I,D).

These results were predicted by past findings on mice injected from birth with anticlass II monoclonal antibodies (mAbs) (*18, 19*) and on animals carrying rearranged T cell receptor (TCR) transgenes from class II-restricted T cell clones (*20, 21*). However, they contradict and prompt one to question some observations on patients with combined immunodeficiency due to a congential defect in class II gene expression (for review, see Ref. 22). Many of these individuals were reported to harbor normal numbers of $CD3^+$ lymphocytes in the periphery and often only slightly reduced numbers of $CD4^+$ cells. One must ask whether the thymi of these patients are truly devoid of MHC class II molecules.

Closer examination of Fig. 1 indicates that there are a few $CD4^+$ T lymphocytes in the peripheral immune system of II^0 mice, usually about 5% the normal numbers. Although these cells express T markers such as CD5 and Thy-1 and display $\alpha:\beta$ TCRs, they are peculiar T lymphocytes by a number of criteria. Most notably, they are preferentially localized in the B rather than the T cell areas of the spleen and lymph nodes (*11*). In addition, an unusually high proportion of them display markers indicative of cells that have been activated (high CD44, LFA-1, and ICAM-1; reduced TCR$\alpha\beta$; low Mel-14, CD45RB, 3G11, and 6C10), although not recently (low CD25 and CD69) (*11*; S. Cardell, unpublished).

Obvious questions arise: Where do these cells originate? What ligands are involved in selecting them? And what activates them? When FITC was injected into thymi of II^0 mice, lymph node $CD4^+$ cells were clearly labeled after 48 hours, indicating that at least some of them are thymically derived (S. Cardell, unpublished). In addition, measurements of their frequency through ontogeny demonstrate that they appear and accumulate in the periphery with the same kinetics as $CD4^+$ cells from normal animals (S. Cardell, unpublished). When the II^0 mice were crossed with animals carrying debilitating mutations in the β_2-microglobulin gene (*23*) or immunoglobulin (Ig) heavy chain locus (*24*), the residual $CD4^+$ population appeared as usual, suggesting that these cells are not selected via MHC class I molecules nor via ligands present on mature B cells, such as Igs or endogenous superantigens (S. Chan, unpublished). It remains possible that the residual $CD4^+$ cells in II^0 mice are selected on nonconventional class II molecules, such as those encoded at H-20 or H-2M loci (*14–16*), or that they are an unselected population. More definitive information about ligands involved in their selection and activation will require their cloning and expansion *in vitro*. Fortunately, they do up-regulate early activation markers, proliferate, and produce IL-2 in response to treatment with anti-CD3 mAbs or bacterial superantigens such as staphylococcal enterotoxin (SE) B (S. Cardell, unpublished).

Whatever its origin and specificity, the small population of $CD4^+$ T cells in II^0 mice is intriguing. Their preferential localization to B cell follicles reminds one of the population of T cells in normal animals that resides in follicles, especially in germinal centers, when they arise (*25, 26*). These cells are evolutionarily conserved, occurring in humans (*27, 28*), rats (*29*), and toads (M. Cooper, personal communication). The human cells seem not to exhibit conventional helper activity, being very poor producers of IL-2 and B cell growth factor (BCGF) (*25, 26*). Interestingly, they often coexpand in patients with B cell chronic

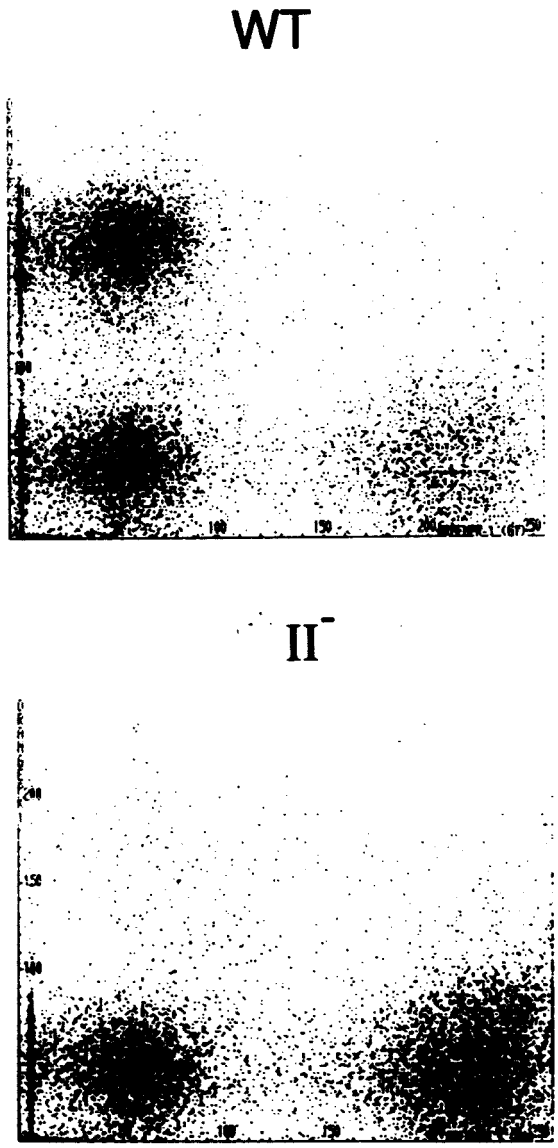

FIG. 1. Depletion of $CD4^+$ T cells in lymph nodes of class II-deficient animals: cells from wild-type (WT) or class II-deficient (II^-) animals, stained with mAbs to CD4 and CD8 and analyzed by flow cytometry.

lymphocytic leukemia or multiple myeloma (*30*), suggesting that they may respond to factors produced by such B cells.

B. $CD8^+$ T Cells

There has been discussion for years about the degree to which MHC class I-restricted $CD8^+$ T cells require input from class II-restricted $CD4^+$ T cells to mature and/or expand (*3, 31*). Most experiments addressing this issue have relied on mAb blockade or depletion of the $CD4^+$ compartment. Unfortunately, these have yielded conflicting results; moreover, criticisms have been raised concerning the effectiveness of such mAb treatments, particularly when important effector cells can be found in environments remote from the circulation. Evidence has recently been presented that a specific $CD4^+$ T cell subset preferentially persists after anti-CD4 mAb treatment (*32*) and that a residual population of $CD8^+$ T cells (*33*) as well as class I-restricted cytotoxic T lymphocyte (CTL) activity (*34*) remain after anti-CD8 treatment. Hence, the II^0 mice provide a valuable system for readdressing this issue.

$CD8^+$ T cells are readily detected in the periphery of II^0 mice; in fact, as seen in Fig. 1, they consistently appear in increased numbers. According to a variety of markers—CD25, CD44, CD45, Mel-14, etc.—they seem quite normal (S. Cardell, unpublished). But can these cells terminally differentiate and function efficiently in the absence of MHC class II-restricted T cell help?

This question has been explored using several viral systems. In the most complete study to date, II^0 mice were infected with influenza A virus, and their ability to clear virus, to mount an *in vivo* CTL response against the class I-restricted nucleoprotein peptide NP 366-374, and to respond to this same peptide after a secondary challenge *in vitro* were evaluated (*35*). When housed under SPF conditions, the animals could clear virus with only a slight delay and were efficient at generating both primary and memory CTL responses. Interestingly, when kept in a conventional animal facility, the primary response was slightly impaired and the memory response substantially reduced. The basis for this difference has not been established experimentally, but one is tempted to suggest that some cells in II^0 mice, perhaps $CD8^+$ T cells, are capable of producing the lymphokines needed for CTL maturation and/or expansion, but that the supply of lymphokines is limited and can be exhausted by the constant (if low level) challenge faced in a conventional animal facility.

Infection of II^0 mice with other viruses has substantiated the conclusion that $CD8^+$ T cells can function in the absence of class II-restricted

$CD4^+$ T cells. Reduced responses were sometimes apparent, but this was not always the case, seeming to depend on the particular virus or assay employed. For example, M. Battegay, R. Zinkernagel, and colleagues (personal communication) found that both mutant and control animals could mount a footpad swelling reaction when challenged intrapedally with the WE strain of lymphocytic choriomeningitis virus (LCMV), although the reaction of mutant mice was somewhat attenuated. Correspondingly, Laufer *et al.* (*36*) saw no significant difference in splenic antivirus CTL responses 7 days after intraperitoneal infection with LCMV-ARM. The latter group also crossed the II^0 line with a strain carrying an LCMV glycoprotein transgene expressed only in pancreatic islet cells. After infection with LCMV, the class II-negative glycoprotein transgenics developed hyperglycemia as fast and as severely as their class II-positive littermates (*36*). Finally, mutant mice were found after vaccinia virus infection to make a primary CTL response that was about five- to seven-fold less vigorous than that of littermate controls (M. Battegay and R. Zinkernagel, personal communication).

Two general conclusions seem merited: (1) $CD8^+$ T cells can function essentially normally in the complete absence of class II-restricted $CD4^+$ T cells; (2) the variability in $CD8^+$ T cell autonomy that was observed previously cannot be attributed just to methods of $CD4^+$ T cell depletion, but must also depend on different systems, assays, and, in particular, conditions of animal housing.

C. $\gamma:\delta$ T Cells

Not much is known about the ligands involved in selecting and activating $\gamma:\delta$ T cells. Two pieces of evidence have implicated MHC class II molecules in positive selection of at least some of them. First, a few cases have been reported of $\gamma:\delta$ T cell clones that can be stimulated by class II molecules, the implication being that these clones might have been selected on the same or similar molecules (*4*, *6*, *37*). Second, it was found that strains of mice displaying the class II E molecule generally have more intraepithelial lymphocytes (IELs) displaying $V\delta4^+$ receptors than strains not expressing this class II molecule. Thus, Lefrançois and colleagues (*5*) hypothesized that E constitutes part of the ligand mediating positive selection of $V\delta4^+$ IELs, actually extrathymically.

Studies on II^0 mice have so far provided no evidence to support a role for class II molecules in shaping the $\gamma:\delta$ T cell repertoire. No changes were observed in the proportion of $\gamma:\delta$ cells in the thymus, lymph nodes, and spleen (*38*; D. Cosgrove *et al.*, unpublished results).

Dendritic epidermal T cells had the usual distribution and density in epidermal sheets from ear, torso, and skin, and expressed Vγ3 as expected (*38*). There were no apparent changes in the γ:δ IEL compartment; in particular, the percentage of cells with $V\delta4^+$ receptors was not reduced (*38, 39*). Schleussner and Ceredig (*39*) directly tested the proposition that E molecules mediate positive selection of $V\delta4^+$ IELs by comparing II^0 mice and E-only mice produced by mating the II^0 line with a transgenic strain carrying the Eα gene (discussed in Section III,A). Their failure to find a difference contradicts the findings of Lefrançois and colleagues (*5*), but is more in line with recent results on a larger panel of E^+ and E^- strains (*7*).

Whether class II molecules are involved in selecting the rare class II-reactive γ:δ clones or whether these clones just exhibit a cross-reactivity remains to be answered. Mating the II^0 line with appropriate strains of γ:δ TCR transgenics should provide a clue.

D. B Cells

MHC class II molecules first appear on rather mature B cells in the mouse, so they have not generally been considered to be crucial for B lymphocyte differentiation. However, treatment of mice from birth with an anticlass II mAb (under conditions thought to be nonlytic) substantially reduce the number of $sIgM^+$ and $sIgD^+$ B cells found in weeks-old animals (*8*). And more recently, an anticlass II mAb and a class II antisense transgene were both found to inhibit B cell maturation at a pre-B stage (*9*).

B lymphocyte differentiation in II^0 mice is surprisingly normal. Minor differences were reported in B cell precursor populations in the bone marrow, but these were not statistically significant (*40*) nor were they confirmed in an extensive four-color cytofluorimetric analysis (W. Müller and K. Rajewsky, personal communication). Initial experiments on mice housed in a conventional mouse facility indicated an abnormally low proportion of IgM^+D^+ cells in the spleen and lymph nodes (*11*) but this is not true of animals kept under SPF conditions (*40;* S. Chan *et al.*, unpublished results). The peritoneal B-1 cell compartment as well as the conventional B-2 cell compartment of mutant mice is essentially normal (*40;* W. Müller and K. Rajewsky, personal communication). The one striking anomaly is the complete absence of germinal centers (*11*). Nevertheless, it is possible to detect abundant IgM^+ and IgG^+ plasma cells in their usual locations (*11*).

That B cell differentiation can proceed to its terminal stage in II^0 mice is further indicated by functional studies. Serum immunoglobulins of the IgM and IgG classes are present at approximately normal

levels, except for IgG_1, which is reduced about 10-fold (*11*, *40*). This isotype has been shown in past experiments to be the most dependent on T cell help, although *in vitro* and *in vivo* experiments have not always provided consistent results (for reviews, see Refs. *41–43*). Responses to type 1 T-independent antigens by mutant mice are very much like those of control animals (*40;* D. Cosgrove *et al.*, unpublished results), as are responses to type 2 T-independent antigens, including levan, dextran, phosphorylcholine, and TNP–Ficoll (*11*, *13*, *40*). In addition, small resting B cells from mutant animals are quite capable of participating in noncognate B–T cell interactions with anti-CD3 preactivated T cells (*40*).

Despite their competency to support terminal B lymphocyte differentiation, II^0 mice are completely unable to make T-dependent antibody responses after injection of complex proteins (*11*, *12*). This was shown after multiple (up to five) injections for keyhole limpet hemacyanin and ovalbumin (*11*). Mutant mice also make almost no antivirus antibodies after LCMV infection (*36*) and are unable to make an IgM-to-IgG switch after infection by vesicular stomatitis virus, a switch known to be crucially dependent on T cell help (M. Battegay and R. Zinkernagel, personal communication). They do make a very low IgM and IgG response to influenza A virus infection, but it is not known whether this is T dependent or independent (H. Bodmer, unpublished).

Finally, a few words about the less commonly studied B cell responses: II^0 mice are capable of making both IgE and IgA antibodies. Serum concentrations of IgE are depressed in some animals, but normal levels of this antibody class are secreted when mutant B cells cooperate *in vitro* with activated T cells (*40*). IgA is found at normal levels in the serum (*40;* C. Benoist, unpublished). Germinal centers with IgA^+ cells are absent from the Peyer's patches of mutant mice, like germinal centers in other lymphoid tissues, but IgA^+ plasma cells are readily apparent in the lamina propria of unstimulated mutant animals (C. Benoist, unpublished).

In short, it seems that MHC class II molecules are not critically involved in B cell differentiation. However, class II-restricted T cell help is absolutely required for certain types of B cell responses.

II. The Thymus

A. The CD4 Single-Positive Compartment

Given the dearth of $CD4^+$ T cells in the periphery (Fig. 1), and considering currently popular models of thymocyte selection (*44*, *45*), one would expect to find very few CD4 single-positive cells in thymi

from II^0 mice. However, as illustrated in Fig. 2, the mutant animals do have a substantial number of such cells. In the many animals analyzed, one routinely observes about 15% of the usual number of CD4 single-positives and sometimes as high as 30% (*11, 46*). A large proportion of these cells expressed CD3 and TCR $\alpha:\beta$ at quite high levels, suggesting maturity. These cells were not observed in the initial analysis of one of the Aβ knock-out lines (*12*), probably due to technical aspects of the cytofluorimetic analysis, but they were seen in the Aα knock-out strain (*13*). Interestingly, careful examination allowed the detection of an analogous population of CD8 single-positive thymocytes in mice lacking MHC class I molecules (*46*); their relative rarity had previously prevented detection (*24, 47*).

An extensive analysis of the CD4 single-positive population in II^0 thymi has highlighted two major characteristics (*11, 46*). First, these thymocytes appear intermediate in maturity between the $CD4^+CD8^+$ and $CD4^+8^-$ cells of wild-type mice. They show clear evidence of having been positively selected: up-regulation of TCR levels, turn-on

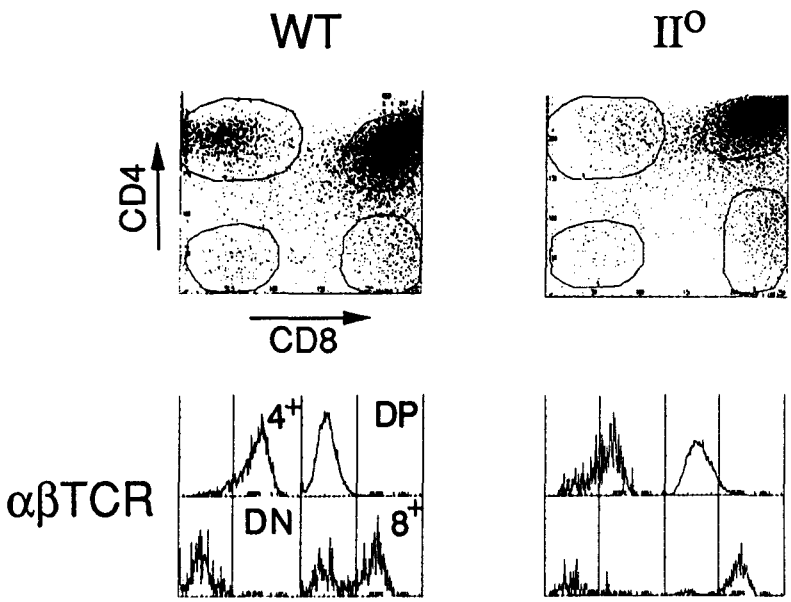

FIG. 2. Altered selection of $CD4^+$ cells in the thymus of class II-deficient mice. Thymocytes from wild-type (WT) or class II-deficient (II^0) animals were analyzed by flow cytometry after staining with mAbs directed against CD4, CD8, and T cell receptor. (Top) Dual-parameter plot of CD4/CD8 staining. (Bottom) TCR intensity histogram for each of the four populations defined above. DN, 4^-8^- double negatives; DP, 4^+8^+ double positives; SP, 4^+8^- or 4^-8^+ single positives.

of CD69, down-regulation of recombination activating gene (RAG) and terminal deoxynucleotidyl transferase (TdT) gene transcripts, and competence to respond to TPA/ionomycin. Yet, they also exhibit features of immaturity: low-level expression of CD8, a cortical localization, and expression of early differentiation markers such as heat-shock antigen and the peanut agglutinin receptor. A similarly schizophrenic population has been observed in recent years in mice that do express class II molecules (*48–55*) and this was reconfirmed in controls in the II^0 studies (*46*).

A second characteristic of the CD4 single-positive population of thymocytes in II^0 mice is that they would seem to have been selected on MHC class I molecules. This was established in two mating experiments (*46*). The II^0 mice were crossed with a strain lacking MHC class I molecules due to a mutation in the β_2-microglobulin gene (*23*), producing doubly deficient animals lacking both class I and class II molecules (discussed in Section III,B). The $CD4^+CD8^{lo}$ population disappeared, indicating its dependence on class I molecules. II^0 mice were also mated to a strain carrying the rearranged TCR genes from a class I-restricted, H-Y-specific T cell clone (*56*), generating II^0 animals with a repertoire highly skewed for a class I-restricted specificity. This specificity was well represented in the $CD4^+CD8^{lo}$ population.

As discussed in more detail elsewhere (*46, 59*), the presence of this CD4 single-positive population in II^0 mice and its characteristics are inconsistent with the previously popular instructional model of positive selection (*44, 45*). This model posited that commitment to the CD4 or CD8 lineage takes place at the $CD4^+CD8^+$ stage of thymocyte differentiation. A double-positive cell expressing an MHC class II-specific TCR would be capable of coengaging class II molecules on stromal cells with its TCRs and CD4 coreceptors. If this occurs, the cell would be instructed to down-modulate its extraneous CD8 coreceptors and proceed with differentiation, rather than die. In an analogous fashion, a double-positive cell displaying a class I-specific TCR could coengage class I molecules with its TCRs and CD8 coreceptors, leading to down-modulation of CD4 expression. This model makes no provision for $CD4^+CD8^{lo}$ thymocytes expressing high TCR levels in mice lacking MHC class II molecules, nor for class I-specific, CD4 single-positive cells. Other data challenging this model have also recently appeared (*58*).

Thus, a new model with a stochastic/selective base has been proposed (see further discussion in Refs. *46* and *51*). It seems that $CD4^+8^+$ thymocytes must effectively engage MHC molecules at least twice before becoming fully mature $CD4^+8^-$ or $CD4^-8^+$ T cells. The first

engagement initiates the positive selection program. It is conditioned by the specificity of the thymocytic TCR and is MHC allele specific; nevertheless, it is nondirectional—either a CD4 or a CD8 single-positive cell can result, regardless of TCR specificity. It is not yet known whether this "hit" requires participation of the coreceptors. The second engagement, requiring participation of the appropriate coreceptor, completes the positive selection program. According to this scenario, cells with either a class II- or I-specific TCR reside in the intermediate $CD4^+8^{lo}$ compartment. The former can differentiate further, but the vast majority of the latter are doomed to die, because they cannot coengage the same MHC molecule with their TCR and coreceptor. It is these latter cells that are detected in thymi of class II-deficient mice. Likewise, cells with TCRs specific for either class I or II molecules reside in the analogous $CD4^{lo}CD8^+$ compartment. Those with a class I-specific receptor continue differentiation, and those with a receptor specific for class II molecules die.

B. Other Compartments

As illustrated in Fig. 2, the three other major thymocyte compartments are readily seen in II^0 mice. $CD4^-CD8^-$ cells occur in normal numbers, and these include both an $\alpha:\beta$ TCR-negative and TCR-positive subset (*11*). $CD4^+CD8^+$ cells are also found at the usual frequency, but they display levels of CD4 elevated 1.5- to 2-fold (*11*, *13*) and levels of TCR increased 2- to 3-fold, on average (*11*). $CD4^-CD8^+$ cells are often, though not always, increased in number, appearing to compensate for decreased numbers of the $CD4^+CD8^-$ counterpart (*11*).

The up-regulation of CD4 and TCR levels on double-positive thymocytes is interesting in terms of past studies by Singer and colleagues. They treated animals or thymus organ cultures with anti-CD4 mAbs and saw a very similar up-regulation of TCR levels (*59–62*). Surprisingly, their parallel experiments with anticlass II reagents showed no such effect (*59–61*). Taken together, the data on II^0 mice and the mAb blocking data indicate that stromal cell class II complexes engage CD4 molecules on double-positive thymocytes and transmit a negative signal that maintains low TCR levels. If and why such a dampening is required for subsequent thymocyte differentiation are unanswered questions (but see further discussion in Section III,A).

C. Reconstituted II^0 Thymi

Thymi from mutant mice can be employed in new approaches to study the role of MHC class II molecules and class II-positive stromal

cells in the selection of $CD4^+$ T lymphocytes. The first *in vivo* and *in vitro* studies along these lines have already been undertaken.

Markowitz *et al.* (*63*) constructed chimeras using irradiated II^0 hosts and donor bone marrow cells from syngeneic wild-type donors. They found the expected defects in the $CD4^+8^-$ thymic and $CD4^+$ peripheral compartments of these chimeras, permitting the conclusion that positive selection of $CD4^+$ T cells requires expression of class II molecules on radiation-resistant cells, presumably cortical epithelial cells in the thymus. It is important to note that an experiment of the same design using irradiated MHC class I-deficient hosts led to the conclusion that positive selection of $CD8^+$ T cells can be mediated, if inefficiently, by class I molecules on hematopoietic cells (*64*). This dichotomy had been noted in the past (*65–68*).

A related question has been explored using an *in vitro* system. It is possible to dissociate fetal thymi enzymatically, allow them to reassociate, and then obtain full thymocyte maturation after some days of culturing (*69*). When chimeric dissociation/reassociations are performed, using Thy-1 allotype-marked II^0 and II^+ thymi, fully mature $CD4^+8^-$ cells appear and they derive from both mutant and wild-type precursors (M. Merkenschlager, unpublished). If chimeras are constructed using graded ratios of II^0 and II^+ thymi, there is a striking linear relationship between the input of II^+ stroma and the output of mature $CD4^+CD8^-$ cells. Even at the highest degree of chimerism tested (75% II^+), the full complement of CD4 single positives was not observed (M. Merkenschlager, unpublished). Thus, positive selection, unlike negative selection (*70*), is not very efficient when the relevant stromal cells are a minority population.

Systems such as these offer much promise for efforts to understand positive selection at the cellular and molecular levels.

III. Beyond the II^0 Line

The information presented here represents a status report on our knowledge of the immune system of II^0 mice. The results have proved interesting and useful, some expected but some a big surprise. The future of the II^0 line may rest largely in exploiting it as a "building block" for other new strains. New genes can be introduced onto the II^0 background or additional genes removed from it. We provide an example of each in the following discussions.

A. E-Only Mice

The majority of mouse strains express both of the conventional MHC class II isotypes, A and E. Strains exist in the laboratory and in the wild

that display A but not E molecules. Since these appear to have quite normal immune competence, one is led to question why strains displaying E but not A molecules have never been observed. Certainly, there have been suggestions in the past that the two isotypes might not be functionally equivalent (*71–78*). This proposition can now be directly tested through the artifical production of E-only mice. A II^0 line was mated to a transgenic strain carrying an Eα transgene on the H-2^b background (*79*); offspring from such a cross include A^+E^-, A^-E^-, and A^-E^+ littermates. The three types of littermates have been compared according to a number of parameters: profiles of peripheral T cells, thymic T cell profiles, CTL responses to influenza A virus infection, serum Igs, antibody responses to complex protein antigens, and TCR repertoire. In general, the A and E molecules are entirely equivalent in their ability to complement the II^0 mutation. However, two subtle differences were observed in A- and E-only mice: (1) the former, but not the latter, down-regulate CD4 and TCR levels on double-positive cells in the thymus, implying effective CD4-MHC class II engagement (as discussed in Section II,B); (2) the two types of animals have subtle differences in the repertoire of $CD4^+$ T cells selected into the periphery—more specifically, differences in the amino acid composition at the first five positions of the CDR3. It is difficult to explain the lack of naturally produced E-only strains on the basis of these subtle dissimilarities. Further studies would seem to be in order.

The ability to complement the II^0 mutation with an Eα transgene has permitted more sophisticated studies with mutated transgenes. Thus, a II^0 line was crossed with previously described strains carrying promoter-mutated genes directing compartmentalized expression of E molecules (*81, 82*). Among the offspring were mice whose thymi expressed this class II molecule essentially only on cortical epithelial cells and others whose thymi displayed it only on the usual contingent of medullary cells. The former, but not the latter, positively selected the $CD4^+CD8^-$ T cell compartment, confirming the critical role of stromal cells of the cortex for this selection event (*83*). This experiment served to generalize some previous studies of similar design but was limited to assaying positive selection of T cells expressing a single transgenic E-restricted TCR (*20*) or a subset of V$\beta6^+$ TCRs (*82, 84*).

B. Double-Deficient I^0II^0 Mice

Mice lacking MHC class I molecules due to a directed mutation in the β_2-microglobulin gene have also been described (*23, 47*), and may be referred to as I^0 mice. By judicious crossing of the class I-negative and class II-negative lines, it is possible to produce littermates of four types—I^+II^+, I^+II^0, I^0II^+, and I^0II^0—which constitute an extremely

useful set of experimental animals. By and large, the double-deficient mice show a phenotype representative of the sum of the single deficiencies: they essentially lack $CD4^+$ and $CD8^+$ cells in the periphery, have almost no fully mature CD4 or CD8 single-positive cells in the thymus, and make no class I- or class II-restricted T cell responses, yet have a rather intact B cell compartment, capable of mounting an efficient antibody response to T-independent antigens (*46, 85*).

This set of MHC-deficient mice has already proved its utility in experiments aimed at elucidating the effector cells and target ligands involved in skin graft rejection. Let us consider, first, deficient mice as skin donors (*85–87;* S. Chan *et al.*, unpublished results). Perhaps not surprisingly, grafts from both I^0 and II^0 mice are rapidly rejected, across either major plus minor or just minor histocompatibility differences. The remaining MHC class I or II molecules must serve as targets for, or present peptides from minor histocompatibility (minor H) molecules to, effector T lymphocytes, consistent with many past results. Rather unexpected, on the other hand, was the rapid rejection of skin from I^0II^0 mice, because these grafts should not contain T cell targets, either as allostimulators or as presenters of minor H peptides. This rejection was clearly demonstrated to be dependent on T lymphocytes, in particular $CD4^+$ T cells, prompting the hypothesis that some nontraditional ligand is involved, perhaps the mysterious H-20 or H-2M gene products (*87*). Whatever the ligand, this rejection phenomenon must serve to caution those aiming to engineer universal donor tissues by abolishing expression of MHC class I and class II molecules.

Experiments on MHC-deficient mice as skin acceptors are less advanced, but the few results already obtained are also intriguing (*85–87;* S. Chan *et al.*, unpublished results). The surprise is that I^0II^0 hosts can rapidly reject grafts differing at major plus minor or just minor histocompatibility loci. The mechanism of this rejection remains undetermined.

IV. The Future

The results summarized in this review attest to the value of II^0 mice for immunologists of diverse interests. Some of the results were expected but nevertheless provide important independent confirmation of fundamental notions—for example, the critical role of class II molecules in the positive selection of $CD4^+$ T cells, or the lack of an important role in B cell differentiation. Other results have been quite a surprise—the existence of an intriguing population of peripheral $CD4^+$ T cells, activated and localized in the B cell follicles, or the

presence of a substantial population of $CD4^{+}8^{lo}$ cells in the thymus, challenging the previously popular instructive model of positive selection. The value of these mutant mice has been enhanced even further by using them as building blocks for new strains with supplementary genes added or additional genes subtracted.

By now, the II^{0} line has been introduced into over 100 laboratories throughout the world. Many studies have been initiated on the role of MHC class II molecules and CD4 T lymphocytes in viral, bacterial, and parasitic infections. Many mating experiments have also been undertaken, among the most promising being the generation of mouse strains expressing only human class II molecules, in particular those implicated in certain autoimmune diseases. The future looks interesting.

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Index

A

B

C

D

E

F

G

H

I

K

L

M

N

P

R

S

T

V