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The diverse roles of J-proteins, the obligate Hsp70 co-chaperone

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Abstract Hsp70s and J-proteins, which constitute one of the most ubiquitous types of molecular chaperone machineries, function in a wide variety of cellular processes. J-proteins play a central role by stimulating an Hsp70's ATPase activity, thereby stabilizing its interaction with client proteins. However, while all J-proteins serve this core purpose, individual proteins are both structurally and functionally diverse. Some, but not all, J-proteins interact with client polypeptides themselves, facilitating their binding to an Hsp70. Some J-proteins have many client proteins, others only one. Certain J-proteins, while not others, are tethered to particular locations within a cellular compartment, thus "recruiting" Hsp70s to the vicinity of their clients. Here we review recent work on the diverse family of J-proteins, outlining emerging themes concerning their function.

Introductory remarks

Molecular chaperones are a ubiquitous class of proteins that interact with short stretches of hydrophobic amino acids typically exposed in partially unfolded proteins. Through such interactions, chaperones function in a broad range of physiological processes, facilitating protein folding, protein translocation across membranes, and remodeling of multimeric protein complexes. Hsp70s and J-proteins (often also referred to collectively as DnaJ-like proteins or Hsp40s), which form obligate partnerships, are among the most ubiquitous of the chaperones. In fact, most eukaryotic and prokaryotic genomes encode both multiple Hsp70s and multiple J-proteins, reflecting the fact that they have evolved to function in such a wide variety of processes. The number of J-proteins, particularly, has expanded with the com-

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plexity of the organism in which they are found. For example, the *Escherichia coli* genome has 6 J-proteins, the yeast *Saccharomyces cerevisiae* genome, 22, and the human genome, approximately 32.

Over the years most research has focused on the Hsp70 component of this chaperone machinery (Bukau and Horwich 1998; Erbse et al. 2004; Slepenkov and Witt 2002). The structure and amino acid sequence of Hsp70s from different organisms and different organelles are remarkably similar. All are composed of a highly conserved N-terminal ATPase domain, followed by a less-conserved peptide-binding domain having a cleft in which hydrophobic stretches of approximately five amino acids interact. Binding and hydrolysis of ATP in the N-terminus regulates the interaction of the C-terminus with unfolded or partially unfolded client polypeptides. ATP hydrolysis stabilizes the interaction with these polypeptide substrates. The essence of all J-protein function is the ability to stimulate the ATPase activity of Hsp70 upon the transient interaction of their highly conserved J-domains with Hsp70's ATPase domain.

In this review, meant to complement earlier reviews that also focused on J-protein function (Cheetham and Caplan 1998; Fan et al. 2003; Walsh et al. 2004), we first discuss the J-domain that is obligatory for the in vivo function of all J-proteins. However, since all J-proteins have domains in addition to their J-domain, the remainder of the review concentrates on recent work aimed at understanding the diverse roles played by these different regions. Some, but not all, of these domains bind client proteins whose transfer to Hsp70s is facilitated by the J-domain. Other domains of J-proteins serve to target the J-protein to a particular location within the cellular compartment in which they function. Below we outline what is known about these additional domains, focusing on the yeast *S. cerevisiae* as a model because of the availability of extensive genomic and genetic analyses.

The J-domain: the common denominator

J-proteins, by definition, contain a conserved, roughly 70-amino-acid signature region, the J-domain, named after the well-studied *E. coli* protein, DnaJ. The DnaJ J-domain contains four α helices, with helices II and III forming a coiled-coil motif around a hydrophobic core (Pellecchia et al. 1996; Fig. 1a). The J-domains of two mammalian J-proteins, human Hdj1 (Qian et al. 1996) and murine polyomavirus tumor antigen (Berjanskii et al. 2000), are remarkably similar. Even the more divergent auxilin J-domain possesses these conserved J-domain features, while also having an N-terminal helix and a long loop inserted between helices I and II (Jiang et al. 2003).

The most highly conserved amino acids of J-domains, the histidine-proline-aspartate (HPD) tripeptide located in the loop between helix II and III, has been shown to be critical for ATPase stimulation in many systems, and thus in vivo function (Feldheim et al. 1992; Tsai and Douglas 1996; Voisine et al. 2001; Wall et al. 1994; Yan et al. 1998). However, additional residues, both within helices II and III and within the intervening loop, are required for the in vivo function of DnaJ (Genevaux et al. 2002). The side chains of these residues and those of the HPD tripeptide are solvent-exposed and oriented in the same direction, and thus possibly form an Hsp70 interaction surface. Indeed, nuclear magnetic resonance (NMR) perturbation mapping of the J-domain of DnaJ in the presence of DnaK indicated a similar negatively charged surface around helix II as the region involved in DnaK interaction (Greene et al. 1998; Fig. 1b).

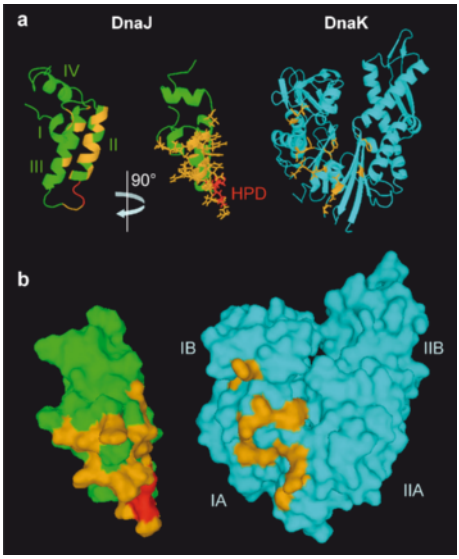

Fig. 1a, b The interaction between the J-domain and the Hsp70 ATPase domain. Ribbon diagram (**a**) and surface map (**b**) of the J-domain of DnaJ (PDB file: 1XBL) (Pellecchia et al. 1996), on the *left*, and the ATPase domain of DnaK (PDB file: 1DKG) (Harrison et al. 1997), on the *right*. Based on the studies of DnaK–DnaJ (Genevaux et al. 2002; Greene et al. 1998; Gässler et al. 1998; Suh et al. 1998) and Ssb-Zuo1 systems (Huang et al. 2005), the residues (or analogous residues in the case of the Ssb-Zuo1 system) found to be important for the interaction between a J-domain and an ATPase domain are highlighted in *orange* with the most critical HPD tripeptide highlighted in *red*. The structures are prepared using PyMOL software (http://pymol.sourceforge.net/). Highlighted residues in DnaK include R167, I169, N170, and T215 (Suh et al. 1998); Y145, N147, D148, E217, and V218 (Gässler et al. 1998), and analogous residues R76, P113, I168, N170, V192, and F200 (Huang et al. 2005), all of which were isolated from genetic mutagenesis studies. The residues highlighted in DnaJ include Y25, R26, H33, P34, D35, R36, N37, F47 (underlined is the HPD motif), from the mutagenesis study (Genevaux et al. 2002); and V12, S13, R19, E20, R22, A24, Y25, K26, R27, L28, M30, Y32, H33, D35, Y54, and T58 that showed a shift greater than 10 Hz in NMR analysis when DnaK was present (Greene et al. 1998)

Consistent with its ability to stimulate Hsp70's ATPase activity, the J-domain of DnaJ interacts with the ATPase domain of DnaK in the presence of ATP (Wittung-Stafshede et al. 2003). However, as is the case with most J-domain:Hsp70 interactions, this association is quite transient (Misselwitz et al. 1999; Suh et al. 1999). Although the exact contact sites between any J-protein and Hsp70 are not known, a region of Hsp70 has been implicated in J-domain interaction in studies of *E. coli* and *S. cerevisiae*. Allele-specific suppressors of the phenotype of *dnaJ-D35N*, which encodes an alteration of the HPD signature motif in its J-domain, were identified in *dnaK*. Three suppressors encoding alterations in subdomain IA of DnaK's ATPase domain were isolated (Suh et al. 1998). In a similar approach, suppressors of a mutation in *ZUO1* (*zuo1-H128Q*), which encodes the J-protein partner of Ssb, the yeast ribosome associated Hsp70, were isolated. Again, alterations were clustered in the AI subdomain (Huang et al. 2005). In addition, site-directed *dnaK* mutant proteins having amino acid alterations in this region were found to have defects in DnaJ interaction (Gässler et al. 1998). Collectively, these residues form a surface on the ATPase domain of Hsp70 (Fig. 1b) with a groove near the nucleotide binding cleft, making it easy to envision binding of a J-domain causing stimulation of ATP hydrolysis by Hsp70.

J-proteins in general protein folding: class I and II

DnaJ was the first J-protein identified and analyzed and still serves as a standard to which other J-proteins are compared. Analysis of its sequence led to the grouping of other J-proteins that contained a glycine-rich and cysteine-rich region adjacent to the J-domain as class I J-proteins, and those that had a glycine-rich region, but lacked the cysteine-rich region as class II (Cheetham and Caplan 1998). This definition was based on obvious sequence similarities, with little understanding of the function of the glycine- and cysteine-rich regions. Below we discuss the current state of understanding of the role of these domains in J-protein function. Recent data also suggest that at least some J-proteins grouped as class I and II have a very similar client protein-binding domain, as even though very low in sequence conservation, they possess a very similar fold. This fold may be common to J-proteins that are involved in general protein folding within the cell, and thus interact with a wide variety of client polypeptides.

Substrate binding: a common fold for general protein folding?

J-proteins, with their Hsp70 partners, are involved in general folding of both newly synthesized and partially unfolded proteins. Evidence exists for such a general function not only for DnaJ working with DnaK in the *E. coli* cytosol, but also for J-proteins in several compartments of eukaryotic cells. For example, Ydj1 and Sis1 of the yeast cytosol work with the Ssa Hsp70s (Aron et al. 2005; Kim et al. 1998; Lu and Cyr 1998a); Mdj1 of the mitochondrial matrix works with the major Hsp70, Ssc1 (Hermann et al. 1994; Krzewska et al. 2001; Rowley et al. 1994); Scj1 of the lumen of the endoplasmic reticulum works with Kar2 (Schlenstedt et al. 1995; Silberstein et al. 1998). Consistent with a general protein-folding role, Ydj1, Sis1, and Mdj1, in cooperation with their Hsp70 partner, are competent to facilitate refolding of denatured substrates such as luciferase in vitro. Orthologs of each of these yeast proteins exist in higher eukaryotes, suggesting that roles in protein folding have been conserved, although as discussed throughout this article, significant functional differences exist among different J-proteins.

Recently the structure of the 25-kDa and 19-kDa C-terminal regions of the class I Ydj1 and class II Sis1 J-proteins, respectively, have been determined (Li et al. 2003; Sha et al. 2000). Although having very limited sequence similarity, the two fragments are remarkably alike in structure. Each contains two domains formed by a sandwich of two β-sheets and a short α-helix, the second of which is followed by sequences important for dimerization (Fig. 2a). Ydj1 was co-crystallized with the peptide GWLYEIS bound in a shallow hydrophobic groove in the N-terminal β-sheet domain (domain I). Sis1 contains a hydrophobic groove at the analogous position in the structure that had been predicted to be the substrate-binding site prior to the determination of the Ydj1 structure (Sha et al. 2000). Despite the similarities between the adjacent β-sheet domains, it is argued that the more C-terminal one is not involved in interaction with client proteins, in part because in the crystal structure the hydrophobic groove of this domain is occupied by a residue from an adjacent β-strand, and thus not available for interactions with client proteins.

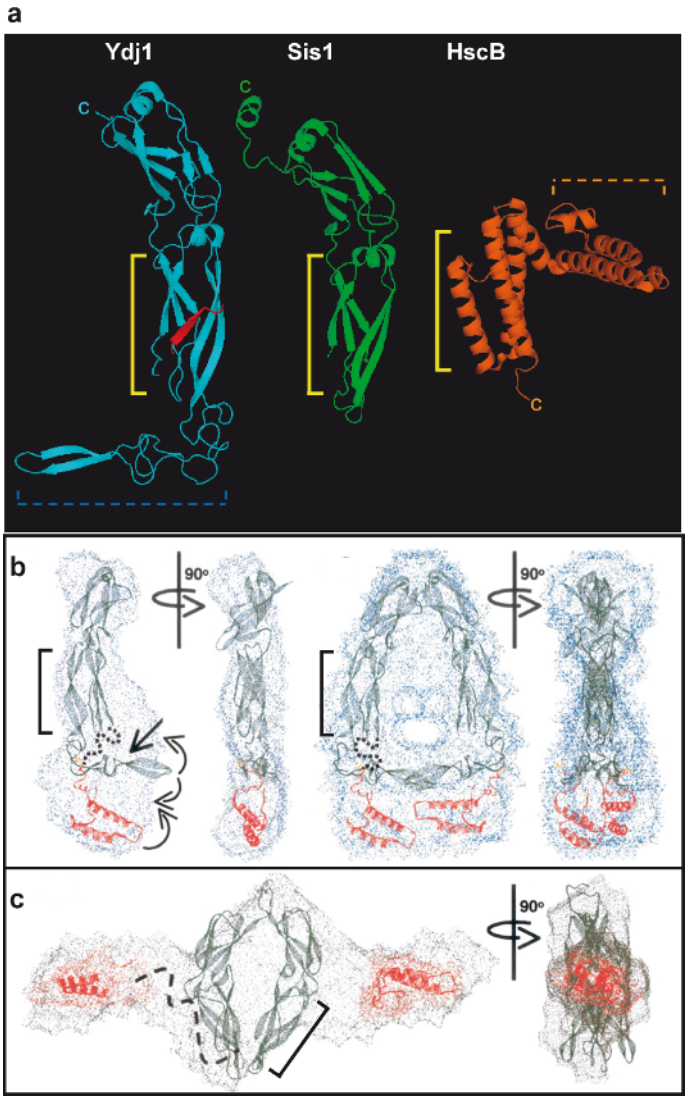

Fig. 2a–c Structural comparison of the substrate binding domains of different J-proteins. **a** Ribbon diagrams of the C-terminal regions of yeast Ydj1 (PDB file: 1NLT) (Li et al. 2003), Sis1 (PDB file: 1C3G) (Sha et al. 2000), and full-length *E. coli* HscB (PDB file:1FPO) (Cupp-Vickery and Vickery 2000) prepared in PyMOL (http://pymol.sourceforge.net/). The crystal structure of Ydj1 contains the bound substrate peptide highlighted in *red*. The proposed substrate binding domains of all the proteins are indicated with the *yellow brackets*. The *dashed blue bracket* indicates Ydj1's cysteine -rich region; the *dashed orange bracket* indicates the J-domain of HscB. The C-terminal end (*C*) of the shown structures of Ydj1 and Sis1 are immediately adjacent to their dimerization domains that are not shown. **b** The low-resolution small-angle X-ray scattering (SAXS) models of monomeric (*left*) and dimeric (*right*) human DjA1, the class I J-protein ortholog of yeast Ydj1. The cysteine-rich domain indicated by the *arrow* can have different angles towards the other domains as shown by the asymmetric packing in the dimer. **c** The low-resolution SAXS model of dimeric human DjB4, the class II J-protein ortholog of yeast Sis1 (**b** and **c** are reprinted from Borges et al. 2005). J-domains highlighted in *red*, the analogous substrate binding domain indicated with *brackets* and glycine-rich regions indicated by *dashed lines*. (Republished with permission of *The Journal of Biological Chemistry*)

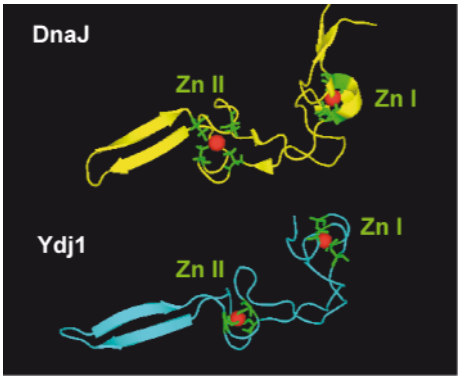

Fig. 3 Ribbon diagram of the Cys-rich domains of DnaJ (PDB file: 1EXK) (Martinez-Yamout et al. 2000) and Ydj1 (PDB file: 1NLT) (Li et al. 2003). The cysteine-rich domains of DnaJ and Ydj1 contain two zinc centers. Coordinated zinc atoms are highlighted in *red*. The cysteine residues involved in the coordination with the zinc atoms are highlighted in *green*

The cysteine-rich zinc center, glycine-rich, and dimerization domains

While Sis1 and Ydj1 show structural similarity, they also differ significantly. Ydj1 has an additional subdomain containing two zinc centers that protrudes from domain I of the C-terminus (Li et al. 2003), such that it is predicted to project into the cleft between the two subunits (Wu et al. 2005) (Figs. 2 and 3). This cysteine-rich domain, which is also present in Scj1 and Mdj1, as well as DnaJ, is the defining feature, along with a glycine-rich region described below, of J-proteins classified as type I (Cheetham and Caplan 1998). In all of these proteins, this region includes four repeats of CXXCXGXG, suggesting a similar fold. Indeed, the structure of the DnaJ subdomain is very similar to that of Ydj1 (Martinez-Yamout et al. 2000). In both cases, two centers are formed, with repeats 1 and 4, and repeats 2 and 3, each coordinating a zinc ion, forming center I and center II, respectively (Fig. 3).

The most quantitative and thorough analysis of the importance of the cysteine-rich zinc binding domains has been carried out with DnaJ (Linke et al. 2003), leading to the view that these zinc centers play different roles: center I in binding to client proteins and center II in facilitating the association of client proteins with DnaK. Disruption of center I by substitution of cysteines by serines dramatically affected binding to a client protein, denatured luciferase, but had little effect on in vivo function. Disruption of center II did not substantially affect luciferase binding or stimulation of DnaK's ATPase activity, but had dramatic effects on the ability of DnaK to bind luciferase and was critical for in vivo function. Similarly, in Ydj1, center II was more important in vivo than center I, especially for substrate transfer to Hsp70 (Fan et al. 2005).

While the function(s) of the cysteine-rich region is becoming clearer, that of the glycine-rich region is still enigmatic, even though its presence is required for classification of a J-protein as a member of class I or II. Typically the glycine-rich region also has a preponderance of phenylalanine residues and thus often referred to as the G/F region. All the J-proteins discussed above contain G/F regions. NMR studies demonstrate that the G/F region of DnaJ (Huang et al. 1999) is very flexible, capable of occupying many different conformational states. However, it does not simply serve as a flexible linker because, as described below, it can include important determinants in the specificity of function of certain J-proteins.

Both Ydj1 and Sis1 are dimers, and in both cases the extreme C-termini are critical for interaction. In the case of Sis1, dimerization occurs via hydrophobic interactions (Sha

et al. 2000). Ydj1 has two modes of interaction between the monomers (Wu et al. 2005). One interaction occurs via hydrophobic interactions very similar to those found for Sis1. The second mechanism utilizes a C-terminal extension, not present in Sis1, which interacts with the more C-terminal β-sheet domain of the other monomer. Conservation of sequences across species suggests that these interactions exist in the orthologs of higher eukaryotes as well.

J-protein/Hsp70 facilitated protein folding: in vitro

Based on extensive in vitro experiments, a model for the function of J-protein/Hsp70 partnership in protein folding has emerged: The J-protein first binds the partially unfolded substrate protein and then to Hsp70, forming a transient tripartite complex dependent upon the J-domain interaction with the Hsp70 ATPase domain. This initial interaction of the unfolded substrate/client protein with the J-protein is predicted to have two functions: (1) prevention of aggregation of the substrate protein, thus maintaining its availability for Hsp70 binding, and (2) facilitating binding of the substrate protein to Hsp70. A broad study of J-protein substrate specificity has only been carried out for DnaJ. Using membrane-bound peptide libraries, a motif consisting of a hydrophobic core of approximately eight residues enriched for aromatic, large aliphatic hydrophobic residues, and arginine was revealed (Rudiger et al. 2001). This binding motif is different from that of DnaK, with DnaJ binding to a broader range of amino acid sequences with less restriction in backbone contacts, although both bind to peptides that are hydrophobic in nature.

The placement of the substrate binding of the J-protein as an important first step in the model of the chaperone cycle comes from the fact that both class I and II J-proteins can bind to denatured proteins such as rhodanase or luciferase and prevent their aggregation (Langer et al. 1992; Lu and Cyr 1998a, b). These experiments have also provided insight into the regions of J-proteins important for substrate binding. For example, suppression of aggregations requires the carboxyl terminus of Ydj1 (Lu and Cyr 1998a). Consistent with this result, deletion of domain I of Sis1's C-terminus is defective in binding denatured luciferase (Aron et al. 2005), suggesting, in line with the structural information described above, that this region is important for binding to unfolded polypeptides.

The ability of J-proteins to function with Hsp70s to promote refolding of denatured proteins in vitro correlated with their ability to bind to the denatured protein. The mutant proteins—defective in their interaction with denatured client protein—are also severely defective in refolding assays. In addition, the fact that at least some class I and II J-proteins are dimers could aid in increasing the affinity for unfolded proteins, as contacts with one client polypeptide could be made by each monomer. Indeed, Sis1 lacking its dimerization domain is unable to cooperate with Hsp70 to refold denatured luciferase, even though it is still capable of stimulating the ATPase activity of Hsp70 (Sha et al. 2000).

If the model described above is correct, and J-proteins first bind to substrate polypeptide, and then target it to Hps70s, an Hsp70/J-protein/substrate complex should exist as an intermediate. Such complexes have been isolated, and do facilitate substrate polypeptide transfer from the J-protein to the Hsp70 (Han and Christen 2003; Gamer et al. 1996). Because of the transient nature of such complexes, the physical arrangement of the components in them remains unclear. However, hints come from recent structural studies that have led to much discussion concerning these interactions. The structure of the dimer formed by the C-terminus of Sis1 suggests not only a bipartite interaction with a substrate protein, but the orientation of the subunits forms a large cleft between them. This cleft has been suggested to

be the docking site for Hsp70 that allows substrate transfer from the J-protein (Landry 2003; Sha et al. 2000). Support for this idea comes from the determination that Sis1 has a second site of interaction with its partner Hsp70 Ssa, in addition to the one via its J-domain. The interaction occurs between the C-terminal 181 residues of Sis1 and the C-terminal 15 amino acid residues of Ssa1, and thus is predicted to "anchor" Hsp70 to facilitate substrate transfer from Sis1 to the peptide-binding domain of Hsp70 (Aron et al. 2005; Qian et al. 2002; Qian et al. 2001). No similar contact has been found for Ydj1 or other type I J-proteins.

None of the high-resolution structures of Ydj1 or Sis1 described above, which were determined by X-ray crystallography, include the J-domain and glycine-rich domain, thus leaving open the question of the quaternary structure of the full-length dimers. Recently, however, low-resolution structural models of the Ydj1 and Sis1 mammalian orthologs—DjA1(Hdj2) and DjB4, respectively—have been generated using small-angle X-ray scattering (SAXS) and other biophysical techniques (Fig. 2b, c). The predicted quaternary structures of these two J-proteins are quite different (Borges et al. 2005). DjA1 forms a compact monomer with both the N- and C-termini aligning with each other. On the other hand, the N-terminal J-domains of the monomers of the DjB4 dimer are in the extremities of the molecule, quite distant from one another, with only the C-termini having contact. There is every reason to believe that Ydj1 and Sis1 have similar differences in structure. These structural models suggest that there is little space between the two substrate binding domains of the class II dimer. Thus, the ability of the C-terminus of an Hsp70 to insert itself deep within the dimer, as predicted based on the crystal structure (Landry 2003; Sha et al. 2000), may not be possible.

J-protein/Hsp70 facilitated protein folding: in vivo

The results of the in vitro experiments described above coalesce into a coherent picture of Hsp70/J-protein-facilitated protein folding requiring interaction of both partners with the client protein. However, the picture in vivo is more complex**.** Unlike alterations in the J-domain, deletion of the substrate binding domains does not typically result in a dramatic loss of function in vivo. A DnaJ C-terminal truncation containing only the J-domain and G/F region supports bacterial growth and is capable of stimulating DnaK's ATPase activity (Wall et al. 1994), even though binding to substrate polypeptide is no longer detectable (Liberek et al. 1995). In addition, both Ydj1 and Sis1 truncations that retain their J-domains and glycine-rich regions are sufficient to substantially rescue the growth defects caused by the absence of the respective full-length proteins (Johnson and Craig 2001; Yan and Craig 1999).

Interpretation of these results is complicated by the presence of more than one J-protein in the cytosol of both *E. coli* and yeast. Analysis of the yeast system revealed that either the substrate binding domain of Ydj1 or Sis1, but not both, was required for cell viability (Johnson and Craig 2001). Thus, substrate binding is likely critical for some roles that type I/II J-proteins play within the cell. However, the in vivo results also suggest that there are many roles for which substrate binding is dispensable (also see the following section). The established protein folding assays likely place demands on the chaperone machinery that is quite stringent, and not necessarily representative of all protein folding/remodeling chores they encounter in the cell.

Specificity of class I/II J-protein function: the Ydj1/Sis1 paradigm

Despite the discussion above that emphasizes similarities between type I and type II J-proteins, we do not want to leave the impression that there are no functional differences between them. The analyses in yeast of Ydj1, a type I, and Sis1, a type II J-protein, underline such differences and provide a window into the interrelationships among functionally similar, but not identical, J-proteins. It has been known for some time that *SIS1* is an essential gene, while *YDJ1* is not, although cells lacking Ydj1 do grow very poorly, especially at 30°C and above (Caplan and Douglas 1991). Only moderate overexpression of Sis1 allows robust growth of *Δydj1* cells, but the reverse is not true (Caplan and Douglas 1991; Yan et al. 1998). Overexpression of Ydj1 cannot rescue a *Δsis1* strain. Thus Sis1 can perform some cellular function(s) that Ydj1 cannot. Remarkably, this specificity in function has been conserved. The Ydj1 ortholog from human cells, Hdj2, can rescue *Δydj1*, but not *Δsis1* cells. The Sis1 human ortholog, Hdj1, can rescue both *Δsis1* and *Δydj1* cells (Lopez et al. 2003).

While the existence of this functional specificity is clear, the biochemical explanation of it is not. However, experiments from several laboratories have yielded intriguing clues. First, surprisingly, the glycine-rich region of Sis1 is competent to define Sis1's specificity. The N-terminal 121 amino acids of Sis1 (Sis1–121) containing the J-domain and 53-amino-acid G/F region is sufficient to rescue the inviability of *Δsis1* cells. A chimera between the J-domain of Ydj1 and the Sis1 G/F segment is also sufficient (Yan and Craig 1999). Comparison of the sequences between the G/F regions of Sis1 and Ydj1 revealed significant similarities, but also two small—12- and 10-amino-acid—"insertions" within the Sis1 sequence. The 10-amino-acid region was found to be critical for function of the G/F region as either its deletion or single amino acid alterations within Sis1–121 rendered it nonfunctional in vivo (Lopez et al. 2003). The J-domain and glycine-rich region of Hdj1, a human class II J-protein, is sufficient for rescue of a *SIS1* deletion as well. However, it should be noted that although the G/F region of Sis1 is sufficient (with a J domain) to carry out Sis1's essential function, other regions of the protein are competent as well (Fan et al. 2004; Yan and Craig 1999). A deletion of the G/F region, which leaves the extended glycine-rich region that has a preponderance of glycine and methionine residues, is able to rescue a *Δsis1* strain.

What is the function of the glycine-rich regions, a hallmark of class I and class II J-proteins? It is conceivable that, at least in the case of Sis1, the glycine-rich region is in fact able to interact directly with substrate polypeptides, like the zinc-center domain of type I J-proteins and the C-termini of both class I and II proteins. However, there are no data to support this idea. A more intriguing possibility is that the glycine-rich region plays a more indirect role in affecting Hsp70's specificity for substrate, either by affecting the conformation of the J-domain itself, or by interacting directly with Hsp70. Consistent with this idea is the fact that the structure of DnaJ's J-domain is different in the presence and absence of the G/F region (Huang et al. 1999). In addition, DnaJ's glycine-rich region has been found to be necessary to "activate" DnaK to bind a client protein, σ32 (Wall et al. 1995). The low-resolution structural information on full-length proteins and information available about the interaction between the C-terminus of Sis1 and Hsp70 described above are consistent with these ideas, leaving open the possibility that via such interactions the glycine-rich regions may help determine Hsp70 activity.

Special folds for special substrates

Any J-protein that lacks a glycine-rich region is placed in class III. Not surprisingly, the structure and function of class III J-proteins are very divergent, since membership is defined only by the presence of a J-domain. However, the class I and class II proteins described above are not the only J-proteins that bind directly to client proteins, as some class III J-proteins do as well. The best-studied are the J-proteins involved in Fe-S cluster biogenesis (HscB/Jac1 in *E. coli* and eukaryotes, respectively) and uncoating of clathrin-coated vesicles (auxilin/Swa2 in higher eukaryotes and yeast, respectively). The role of these J-proteins is likely biochemically similar to the type I proteins, since they stimulate Hsp70's ATPase activity, bind polypeptide substrates, and then—via interaction with Hsp70s—facilitate their transfer to the Hsp70 upon ATP hydrolysis. However, in each of these cases, evidence suggests that each binds only to a single substrate, and that the substrate is a folded protein. Thus, in these cases, chaperone action is not involved in the general folding of client proteins into their tertiary structure but rather in more specific conformational changes. Whether this is also the case for other type III J-proteins that might bind client proteins remains to be seen. Regardless, such specialized chaperones do provide easily accessible experimental systems for sophisticated analyses of J-protein:Hsp70 chaperone machineries both in vitro and in vivo.

Fe-S center biogenesis

Both eukaryotes and prokaryotes have specialized systems for the assembly of Fe-S centers, an essential prosthetic group for certain proteins (Johnson et al. 2005; Lill and Muhlenhoff 2005). Specialized Hsp70:J-protein machineries, called Ssq1:Jac1 and HscA:HscB in yeast and *E. coli*, respectively, are an important part of this process (Craig and Marszalek 2002). Fe-S centers are transiently assembled onto a highly conserved scaffold protein Isu (IscU in bacteria), prior to transfer to apoproteins. Isu/IscU is the only known substrate for this chaperone system. A peptide array performed with the *E. coli* system identified a short amino acid sequence, LPPVK—situated in a loop between two α-helices in the folded protein—as the Hsp70 interaction site (Hoff et al. 2002). This same highly conserved sequence is also important for Hsp70 binding in the yeast system (Dutkiewicz et al. 2004; Ramelot et al. 2004). However, the study failed to identify any sequence of IscU that might be important for binding to HscB, suggesting that HscB does not interact with a linear amino acid segment of Isu/IscU, but rather recognizes a structural motif present on the surface of the folded protein (Hoff et al. 2002).

Despite the specialized nature of this system, the partnership of the Hsp70 and J-protein in Fe-S center biogenesis has the hallmarks of the more general class I and II systems. Jac1/HscB stimulates the ATPase activity of Ssq1/HscA. Jac1/HscB also increases the affinity of Isu/IscU for its Hsp70 partner through formation of an Hsc20–IscU complex, suggesting "targeting" of the client to Hsp70 as described above ("J-protein/Hsp70 facilitated protein folding: in vivo") (Dutkiewicz et al. 2003; Hoff et al. 2000). In vivo evidence for such targeting comes from the yeast system. Overexpression of Jac is capable of overcoming defects in the Hsp70/Isu interaction, suggesting that complex formation between Jac1 and Isu can be important in vivo (Knieszner et al. 2005). However, kinetic analysis of the ability of Isu and Jac1 to stimulate Ssq1's ATPase activity suggests that targeting is not necessary under many conditions; Isu can also interact directly with Ssq1 without first forming

a complex with Jac1 (Knieszner et al. 2005). This flexibility in the mode of interaction of a J-protein, Hsp70, and client protein is reminiscent of the finding that the J-domain/glycine-rich domain fragments of class I and class II J-proteins retain significant activity in vivo.

The structure of HscB has been determined, representing the only high-resolution structure of a full-length J-protein. In addition to a typical N-terminal J-domain, HscB has a C-terminal domain consisting of a three-helix bundle in which two of the helices constitute an anti-parallel coiled-coil (Fig. 2a). Thus, the substrate binding domain of HscB bears no structural resemblance to the functionally analogous domain of class I and II J-proteins. HscB does not appear to contain a hydrophobic pocket for binding substrate proteins (Cupp-Vickery and Vickery 2000). Perhaps this absence of an obvious binding cleft is not surprising, as HscB interacts with a single folded polypeptide. Interestingly, the overall structure of HscB appears to be very rigid, leading to predictions that HscB could position IscU precisely to foster interaction of the LPPVK of IscU with HscA, situated by the interaction of the J-domain with the ATPase domain of HscA. Such rigidity is in stark contrast to the flexible nature of the glycine-rich regions that link the J-domain to the substrate-binding domain in class I and II J-proteins.

Uncoating of clathrin-coated vesicles

Membrane vesicles provide an important means of transport of components across the plasma membrane and within cells. Disassembly of the support lattice surrounding a vesicle is an important step in vesicle trafficking. The coat of one important class of vesicles is composed of clathrin heavy and light chains that interact to form three-legged (triskelion) structures, which in turn interact to form the lattice (Brodsky 2004). The uncoating of mammalian clathrin vesicles requires the J-protein auxilin partnering with the general cytosolic Hsp70, Hsc70 (Holstein et al. 1996; Ungewickell et al. 1995). In addition to its J-domain, auxilin contains a region that binds to the clathrin heavy chain. Like a typical J-protein:Hsp70 partnership, the J-domain of auxilin stimulates the ATPase activity of Hsc70, facilitating its binding to the clathrin/auxilin complex associated with the vesicle, destabilizing the interaction between triskelia.

The fact that auxilin and Hsc70 can drive the uncoating of clathrin-coated vesicles (CCV) has been known for some time (Braell et al. 1984; Ungewickell et al. 1995). Understanding the conformational changes that destabilize the interactions between the triskelia of the lattice have been much more challenging. Recently, however, new structural information further defines the lattice structure itself and the site of auxilin binding, allowing more specific models about the mode of molecular chaperone action in uncoating to be put forth (Fotin et al. 2004a, b; Gruschus et al. 2004; Smith et al. 2004). Auxilin binds near the proposed vulnerable "ankles" of the clathrin triskelia, close to one site of interaction between them. The relative orientation of these "ankles" in the triskelia is different when auxilin is bound and when not, leading to the idea that this conformational change coupled with the recruitment of Hsc70, which can also bind to the clathrin heavy chain, results in destabilization of the lattice.

While the vast majority of the analysis of CCVs has been carried out in mammalian systems, *S. cerevisiae* has an auxilin ortholog, Swa2/Aux1. Disruption of the *SWA2* gene has similar phenotypic effects to disruption of the gene encoding clathrin (Gall et al. 2000; Pishvaee et al. 2000). Similar to auxilin, Swa2/Aux1 contains both a J-domain and a clathrin binding domain. Although the J-domain sequences of auxilin and Swa2/Aux1 are conserved (39% identity), no significant sequence similarity exists between their clathrin binding do-

mains. Nevertheless, GST–Swa2/Aux1 fusions can pull down clathrin from yeast cytosolic extracts, and Swa2/Aux1 can substitute for auxilin in recruiting Hsc70 to CCVs, and promote uncoating of these vesicles in vitro (Gall et al. 2000; Pishvaee et al. 2000). Swa2/Aux1 has also been shown to stimulate the ATPase activity of yeast cytosolic Hsp70 Ssa1, the ortholog of mammalian cytosolic Hsc70, and thus is likely be its in vivo partner (Gall et al. 2000). Thus, Swa2/Aux1 likely functions in yeast, as auxilin does in mammals, in the disassembly of clathrin from clathrin-coated vesicles budded off the plasma membrane, even though the auxilin/Swa2 clathrin binding domains show little sequence similarity.

Tethering of J-proteins to particular subcellular locations

Some J-proteins can fulfill their biological function only if they are targeted to a particular location within the cell. In some cases this simply means targeting to a particular cellular compartment, similar to other nuclear encoded organellar proteins [i.e., the mitochondrial matrix or the lumen of the endoplasmic reticulum (ER)]. However, there are a number of examples of more precise localization within a cellular compartment. We discuss examples of localization to two different cellular structures: membranes and ribosomes.

Membrane localization

J-proteins have been found to associate with specific cellular membranes by a variety of means. In yeast there are J-proteins that are transmembrane proteins having globular domains on both sides of the membrane. In addition, there is an example of a J-protein that undergoes the post-translational addition of a farnesyl anchor that renders it membrane-associated, as well as a J-protein having a tail-anchor that allows post-translational insertion into the ER membrane.

Sec63 and Pam18 are examples of transmembrane J-proteins. Sec63 of the ER membrane and Pam18 of the inner mitochondrial membrane are both involved in the translocation of proteins from the cytosol through the translocation channel of their respective membranes. Both are not only transmembrane proteins but are physically associated with the translocons, with their J-domains extending into the lumenal space of these compartments (Corsi and Schekman 1997; D'Silva et al. 2003; Lyman and Schekman 1997; Mokranjac et al. 2003; Truscott et al. 2003). Yeast mitochondria also contain a J-protein closely related to Pam18, Mdj2, which is a component of the mitochondrial inner membrane, and thought to function in the import process as well (Westermann and Neupert 1997). The precise positioning of the J-domain allows efficient partnering with the respective organellar Hsp70, Kar2 and Ssc1. In particular, the post-translational import of proteins puts exceptional demands on the chaperone system, requiring effective interaction of Hsp70 with the translocating polypeptide at the import channel (D'Silva et al. 2004). In addition, Pam18 and Sec63 have domains that extend into a cellular space different from that occupied by the J-domain: into the cytosol in the case of Sec63, and into the intermembrane space of mitochondria in the case of Pam18 and Mdj2. Presumably these domains play roles in the import process, helping to coordinate interactions across the membrane.

Ydj1 is an example of a J-protein that is post-translationally modified, having a farnesyl group at its C-terminus (Caplan et al. 1992). This farnesyl group is added at a signal typical for such modification, a CaaX box motif, with "a" indicating an aliphatic amino acid. Far-

nesylated Ydj1 is associated with the cytosolic face of the ER membrane. This localization, however, does not appear to be essential for Ydj1 function under normal growth conditions. Alteration of the conserved cysteine in the CaaX box generated a mutant Ydj1 that cannot be farnesylated in vitro and leads to a temperature sensitive phenotype in vivo (Caplan et al. 1992). In addition, *E. coli* DnaJ does not contain a farnesylation signal, and can substitute for Ydj1 at 30°C, but not 37°C. Presumably, a function of Ydj1 at the membrane is more important at higher than at lower temperatures. Such functions could involve post-translational translocation into the ER, facilitating folding of membrane proteins or degradation of misfolded proteins extruded from the ER (Becker et al. 1996; Huyer et al. 2004; McClellan and Brodsky 2000; Meacham et al. 1999).

Hlj1p is a tail-anchored membrane protein (High and Abell 2004) with its J-domain residing in the cytosol (Beilharz et al. 2003; Youker et al. 2004). Ydj1p and Hlj1p, both of which have a cytosolic J-domain and are tethered to the ER membrane, were recently found to function redundantly with the cytoplasmic Hsp70, Ssa, to facilitate the degradation of cystic fibrosis transmembrane conductance regulator (CFTR) (Youker et al. 2004), underscoring the idea that localization of J-proteins to particular subcellular positions is functionally important.

Ribosome association

Tethering molecular chaperones in close proximity to the site where they are extruded from the ribosome during synthesis can serve to prevent aggregation and promote folding of these newly synthesized proteins. All organisms appear to have ribosome-associated chaperones (Craig et al. 2003; Deuerling and Bukau 2004). Prokaryotes have a ribosome-associated member of the peptidyl-prolyl isomerase family, trigger factor. During eukaryotic evolution, ribosome-associated J-proteins have been conserved (Bukau 2005; Hundley et al. 2005), called Zuo1 in yeast and Mpp11 in human cells. Yeast Zuo1 is the J-protein partner of the specialized ribosome-associated Hsp70 Ssb (Huang et al. 2005). Since Ssb can be crosslinked to nascent polypeptide chains that extend only ten or so amino acids into the cytosol beyond the polypeptide exit tunnel of the ribosome, both Ssb and Zuo must interact with the ribosome in close proximity to the exit site (Hundley et al. 2002). Zuo1 likely binds to the ribosome, at least in part, via interactions with ribosomal RNA (Yan et al. 1998). An internal 80-amino-acid segment of Zuo1 has a high propensity of positively charged residues that are required both for association with the ribosome and the ability to bind RNA in vitro. Mpp11, the human ortholog of Zuo1, is also ribosome-associated, even when ectopically expressed in yeast cells. Mpp11 also contains a positively charged region, and can compete with Zuo1 for ribosome binding, indicating overlapping binding sites and a conserved mode of ribosome association for the two proteins. In yeast, Mpp11 can partially rescue phenotypes caused by the deletion of *ZUO1* (Bukau 2005; Hundley et al. 2005).

Mtj1p is another particularly intriguing mammalian ribosome-associated J-protein. It associates with membranes, as well as ribosomes (Dudek et al. 2002). Mtj1p, which has no obvious ortholog in yeast, has a single transmembrane domain that spans the ER membrane. Its J-domain, which extends into the ER lumen, interacts with the ER lumenal Hsp70, BiP. The large cytosolic domain of Mtj1p interacts with both translating and nontranslating ribosomes. Mtj1p is proposed to function during cotranslational protein transport into ER to recruit both the ribosome and BiP to the translocon complex. Mtj1p could participate in facilitating the handover of nascent polypeptides from the signal recognition particle (SRP) to

the translocon complex, transmitting signals from the ribosome to BiP, or regulating lumenal gating of the translocon (Dudek et al. 2002).

Multiple J-proteins can function with a single Hsp70

In most organisms the number of J proteins exceeds the number of Hsp70s. Thus, at least some Hsp70s must partner with more than one J-protein (Fig. 4). In the simplest examples, different J-proteins function with the same Hsp70 because of their targeting to different places within a cellular compartment. In such cases, there is no evidence that there are any fundamental biochemical differences between the J-protein and Hsp70 activities. Simply, the two proteins are able to be in the same place at the same time. The yeast mitochondrion serves as an example. As discussed above ("Substrate binding: a common fold for general protein folding?"), Ssc1 is the major Hsp70 of the mitochondrial matrix, making up about 1–2% of total mitochondrial protein. Approximately 10% of Ssc1 is tethered to the translocation channel via interaction with the peripheral membrane protein Tim44, and functions with the J-protein Pam18, which is independently tethered to the channel (Mayer 2004). The remaining 90% is soluble in the matrix, where it partners with the class I J-protein Mdj1 in protein folding and quality control of protein degradation (Liu et al. 2001; Wagner et al. 1994).

In some cases the J-protein itself is thought to be the "recruiting factor" for the Hsp70. In the case of Sec63, the translocon-associated J-protein of the ER, the association of the lumen Hsp70, Kar2, with the translocon is dependent upon Sec63 (Corsi and Schekman 1997). The yeast cytosol has similar examples. As discussed above ("Membrane localization"), Hlj1, a tail-anchored J-protein of the ER membrane with its J-domain facing the cytosol, functions with the general cytosolic Hsp70s of the Ssa class. There is no evidence for direct targeting of Ssa to the ER membrane, so it is likely that the presence of the J-domain itself is sufficient to recruit Hsp70. Similar statements can be made concerning the portion of Ydj1 that is membrane localized due to its farnesylation.

The cytosol is more complex than either the mitochondria or ER in regards to Hsp70:J-protein function, based simply on the sheer number of J-proteins: 11, with 5 of them being class I or II J-proteins (reviewed in Walsh et al. 2004). Although the identity of their Hsp70 partners is currently unknown, it is likely that most, if not all, are eventually shown to work with Ssa. Recently, it was reported that the human homolog of ribosome-associated Zuo1, Mpp11, functions with Ssa1-the ortholog of mammalian Hsc70, but not Ssb1 when expressed in yeast (Hundley et al. 2005). Since Ssb orthologs are not present outside of fungi, Mpp11 likely functions with Hsc70 in mammalian cells. Consistent with that idea, Mpp11 is able to stimulate Hsc70's ATPase activity. Therefore, it is possible that even more J-proteins work with Hsc70 in higher organisms than work with Ssa in yeast.

The discussion in this section has emphasized the recruitment of Hsp70s to particular sites within cellular compartments by different J-proteins. However, there is evidence that J-proteins can play a more mechanistic role in determining the activity of the Hsp70:J-protein machinery than merely helping to position Hsp70 at a particular site of action. The first hint of such a possibility came from in vitro experiments indicating that interaction of a J-domain with Hsp70 could allow binding of Hsp70 to client proteins to which they would not bind on their own (Misselwitz et al. 1998). In vivo experiments also indicate mechanistic complexity. As discussed above ("Substrate binding: a common fold for general protein folding?" and "Specificity of class I/II J-protein function: the Ydj1/Sis1 paradigm"), both Ydj1 and

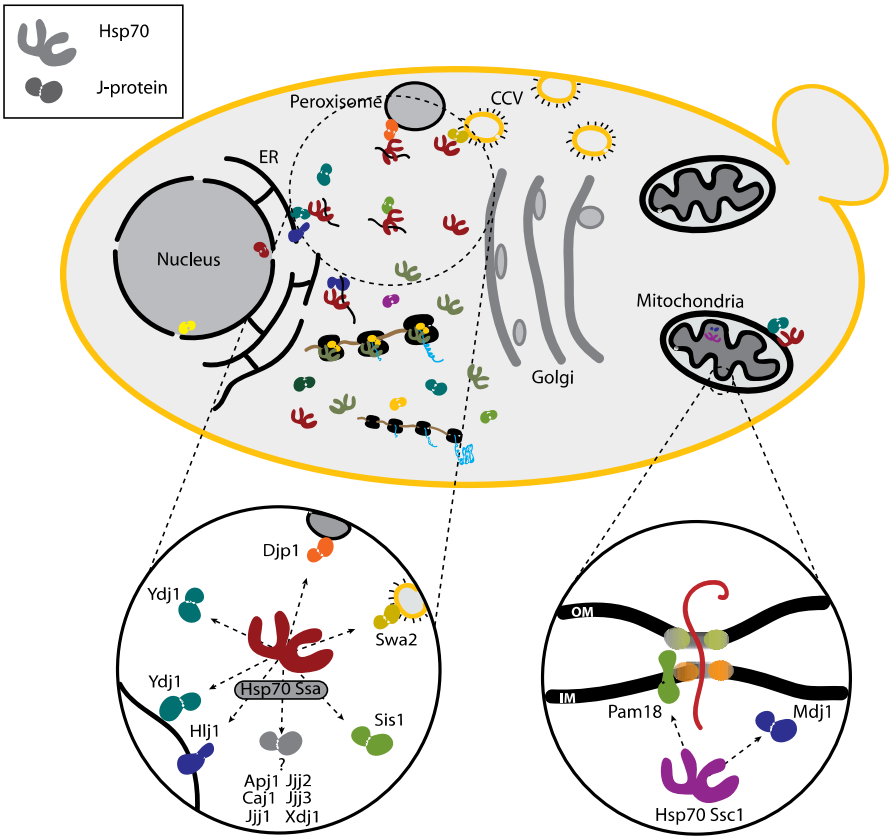

Fig. 4 Multiple J-proteins function with a single Hsp70. Cellular overview representing some of the complexity of Hsp70 and J-protein distribution in cellular compartments of *S. cerevisiae* (*top*). At least five J-proteins are known to interact with the Hsp70 Ssa (*bottom left*), allowing it to function in a variety of cellular processes. Of the five J-proteins, three (Ydj1, Djp1, and Hlj1) are at least partially membrane associated, with their J-domain exposed to the cytosol and available to effectively interact with and stimulate the ATPase activity of Ssa. Six other J-proteins, Apj1, Caj1, Jjj1, Jjj2, Jjj3, and Xdj1 are also possible Ssa partners, based on their cellular localization and sequence similarity to the known J-protein partners of Ssa. The mitochondrial matrix (*bottom right*) serves as another example of a single Hsp70 partnering with more than one J-protein. Both the J-proteins Mdj1 and Pam18 interact with and stimulate the ATPase activity of the Hsp70 Ssc1. Mdj1 is a soluble protein of the mitochondrial matrix, and functions with Ssc1 in general protein folding. Pam18 spans the inner mitochondrial membrane with its J-domain exposed to the matrix lumen, and functions with Ssc1 in translocation of proteins into the mitochondrial matrix. Another J-protein, not illustrated here, Mdj2, is also localized to the inner membrane, with its J-domain exposed to the matrix lumen, and is predicted to function with Ssc1. *CCV* clathrin-coated vesicle, *ER* endoplasmic reticulum, *OM* outer membrane, *IM* inner membrane

Sis1 are soluble J-proteins of the cytosol that partner with Ssa, but Sis1 carries out distinct functions from Ydj1. Importantly this specificity resides in a region of Sis1, the glycine-rich region, not implicated in binding directly to client proteins (Yan and Craig 1999). How common such specificity of function might be, independent of direct binding to substrate proteins, remains to be seen. With the large number of J-proteins present, particularly in higher organisms, more examples may be uncovered.

Degenerate J-proteins

The presence of an HPD tripeptide is an obligate part of the definition of a J-domain because of the critical nature of these amino acids. However, genomes contain sequences that show similarities to J-domains, but lack some or all of the amino acids of the conserved HPD tripeptide. The *S. cerevisiae* genome has three such sequences, Pam16, Jpl1, and Jpl2 (Walsh et al. 2004). Jpl1 and Jpl2 are uncharacterized, but recent work has begun to elucidate the function of Pam16 (Frazier et al. 2004; Kozany et al. 2004; Li et al. 2004). Not surprisingly, Pam16, which has orthologs in higher eukaryotes, appears unable to stimulate the ATPase activity of Hsp70. Similar to Pam18, the J-protein partner of Ssc1, Pam16 has a membrane association domain followed by a J-like domain that is exposed to the mitochondrial matrix. Pam16, an essential protein, forms a heterodimeric complex with Pam18. The J-domain of Pam18 and the J-like domain of Pam16 are sufficient for heterodimer formation, the only known case in which J-domains dimerize. Heterodimer formation is essential, as its destabilization has deleterious consequences on protein import (D'Silva et al. 2005).

A number of possible functions of Pam16 have been proposed, including a "structural" function positioning Pam18 in the correct location for import, and/or a regulatory function, controlling the activity of Pam18 (Frazier et al. 2004; Kozany et al. 2004; Li et al. 2004). Some models, such as the idea that simply the modest reduction of Pam18's ability to stimulate Ssc1's ATPase activity when in a heterodimer with Pam16 is a critical function of Pam16, is likely to be incorrect. When an active J-domain is substituted for an inactive "J-like" domain in Pam16, no in vivo effect is observed (D'Silva et al. 2005).

More likely, Pam16 serves to correctly position Pam18 at the translocon so that it can effectively stimulate Ssc1's ATPase activity, with the N-terminus playing this critical role. *PAM18* and *PAM16* may have arisen from the duplication of a gene encoding a single J-protein that functioned at the import channel as a homodimer. Over time, one retained an active J-domain, the other maintained an N-terminal domain that correctly tethered the complex to the import channel. Both, however, were co-selected to maintain the stability of the heterodimer. It will be interesting to know whether Pam16 serves as a paradigm for other J-like proteins, or whether they have evolved to fulfill very different functions.

Summary

Strides have been made in understanding the diversity of J-proteins and their partner Hsp70s, and the roles they play within the cell. However, many questions remain. Why are there so many different J-proteins? In the yeast cytosol there are three class I (Ydj1, Xdj1, Apj1) and two class II (Sis1 and Djp1) J-proteins. How do they differ in function? Indeed, what are the mechanistic differences between class I and class II J-proteins? Differences in substrate binding are evident, but likely not the whole story, as analysis of Sis1 and Ydj1 indicate.

Three substrate binding domains have been defined, the ones found in class I/II general protein folders and HscB/Jac1, and auxilin of the specialized class III (Table 1). Do others exist? There are regions of many J-proteins whose role at this point remains completely undefined and may well be substrate protein binding domains, such as the dispensable C-terminal domain of Zuo1. Alternatively, are most class III J-proteins simply J-domains tethered to particular locations within the cell? How common is targeting of client proteins by J-proteins to Hsp70s in vivo, or is this activity more prevalent in the in vitro assays we com-

Table 1 *S. cerevisiae* J-proteins classified according to predicted structure and related function[1]

J-protein	Localization	Hsp70 partner
Common fold/general substrate binding[2]		
Sis1	Cytosol	Ssa
Ydj1	Cytosol	Ssa
Djp1	Cytosol/peroxisome	Ssa
Apj1	Cytosol	Ssa?
Xdj1	Cytosol	Ssa?
Mdj1	Mitochondria	Ssc1
Scj1	ER	Kar2
Special fold/special substrate[3]		
Swa2	Cytosol/CCV	Ssa
Jac1	Mitochondria	Ssq
Unknown substrate		
Zuo1	Cytosol/ribosome	Ssb
Jem1	ER	Kar2
Predicted not to bind substrate		
Pam18	Mitochondria	Ssc1
Sec63	ER	Kar2

[1] Some J-proteins of *S. cerevisiae* have been omitted due to lack of information

[2] Structures of the C-terminal domains of Sis1 and Ydj1 involved in substrate binding have been determined (Li et al. 2003; Sha et al. 2000). Other J-proteins listed here are predicted to have protein-binding domains with a similar fold to Sis1 and Ydj1

[3] Clathrin is a substrate of Swa2. Isu is a substrate of Jac1

monly use? We await the answers to these and other questions concerning the function of these intriguing and complex classes of chaperones. End Grabbed content

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Role of the Mediator complex in nuclear hormone receptor signaling

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Abstract Mediator is an evolutionarily conserved multisubunit protein complex that plays a key role in regulating transcription by RNA polymerase II. The complex functions by serving as a molecular bridge between DNA-bound transcriptional activators and the basal transcription apparatus. In humans, Mediator was first characterized as a thyroid hormone receptor (TR)-associated protein (TRAP) complex that facilitates ligand-dependent transcriptional activation by TR. More recently, Mediator has been established as an essential coactivator for a broad range of nuclear hormone receptors (NRs) as well as several other types of gene-specific transcriptional activators. A single subunit of the complex, MED1/TRAP220, is required for direct ligand-dependent interactions with NRs. Mediator coactivates NR-regulated gene expression by facilitating the recruitment and activation of the RNA polymerase II-associated basal transcription apparatus. Importantly, Mediator acts in concert with other NR coactivators involved in chromatin remodeling to initiate transcription of NR target genes in a multistep manner. In this review, we summarize the functional role of Mediator in NR signaling pathways with an emphasis on the underlying molecular mechanisms by which the complex interacts with NRs and subsequently facilitates their action. We also focus on recent advances in our understanding of TRAP/Mediator's pathophysiological role in mammalian disease and development.

Introduction

Initiation of transcription on eukaryotic protein-encoding genes involves the assembly of RNA polymerase II (RNA pol II) and a group of general transcription factors (TFII-A, -B, -D, -E, -F, and -H) into a functional preinitiation complex (PIC) at core promoter elements (Roeder 2005). Activation of transcription by gene-specific activators involves the recruitment of coregulatory factors that locally remodel chromatin and facilitate functional PIC

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assembly. The largest known family of eukaryotic activators comprises the nuclear hormone receptors (NRs). NRs are ligand-activated transcription factors that play essential roles in nearly every aspect of vertebrate development and adult physiology (Aranda and Pascual 2001; Mangelsdorf et al. 1995). In the presence of cognate ligand, NRs activate transcription by recruiting distinct types of coactivator complexes to target gene promoters.

Some NR coactivators act by covalently modifying core histones (Tsai and Fondell 2004) while others rearrange higher ordered chromatin structure (Belandia and Parker 2003). In contrast, the Mediator complex functions by directly facilitating the recruitment and activation of RNA pol II and the general transcription apparatus at NR target genes. First identified as a positive transcriptional activity that copurifies with the thyroid hormone receptor (TR) from mammalian cell extracts (Fondell et al. 1996), Mediator has since been recognized as a broadly utilized coactivator complex for a wide range of NRs and other types of transcriptional activators. In this review, we will summarize the physiological role of Mediator in NR-mediated gene expression with an emphasis on: (1) how the complex is targeted to NRs in the presence of ligand, (2) how the complex facilitates transcriptional regulation of NR-target genes, and (3) the role of the complex in mammalian development and disease.

Nuclear hormone receptor signaling pathways: an overview

NRs are "ligand-activated" transcription factors. The ligands for NRs are lipophilic compounds that include steroids, retinoids, thyroid hormone (T3), and vitamin D3. NRs specifically bind to DNA at promoter-proximal sequences termed hormone response elements (HREs). In general, NRs activate transcription in the presence of cognate hormone, whereas some NRs can also repress transcription in its absence. NRs have a modular structure that consists of three functional domains (Fig. 1a). The first is a highly conserved DNA-binding domain (DBD) composed of two "zinc finger" motifs that mediate specific HRE recognition (Aranda and Pascual 2001). The second domain is a poorly conserved amino-terminal region that, at least for some NRs, contains an autonomous activation function 1 (AF1) (Tsai and O'Malley 1994). The third is a carboxy-terminal ligand-binding domain (LBD) that contains a dimerization surface and an additional activation function 2 (AF2). The latter is essential for ligand-dependent transcriptional activation (Moras and Gronemeyer 1998). The AF2 "core-domain" contains a highly conserved amphipathic α-helical motif that is present in nearly all transcriptionally active NRs (Barettino et al. 1994; Danielian et al. 1992; Durand et al. 1994; Saatcioglu et al. 1993) and serves as a binding site for coregulatory factors (see below).

NRs can generally be divided into two classes based on their ligand-binding and DNA-binding properties (Fig. 1b). Class I comprises receptors for known steroid hormones including glucocorticoids, mineralocorticoids, progesterone, androgens, and estrogen (GR, MR, PR, AR, and ER, respectively) (Tsai and O'Malley 1994). The steroid hormone receptors function as ligand-induced homodimers that bind to HREs in which the DNA half-sites are organized as inverted repeats. Class II includes receptors for nonsteroid ligands including T3, retinoic acid, vitamin D3, prostanoids, and farnesoids (TR, RAR, VDR, PPAR, and FXR respectively) (Aranda and Pascual 2001; Mangelsdorf and Evans 1995). In contrast to the homodimerization observed with class I receptors, class II NRs heterodimerize with the retinoid X receptor (RXR) and characteristically bind to direct repeats of the HRE core half-site, AGGTCA, although some can bind to symmetrical repeats. In general, the spacing

B.

Class I (steroid)		Class II (non-steroid)		Orphan
Receptor	Ligand	Receptor	Ligand	Receptor
GR	cortisol	TR α,β	thyroid hormone	HNF4 α,β
ER α,β	estrogen	VDR	1,25-dihydroxy vitamin D3	ROR α,β,γ
PR	progesterone	RAR α,β,γ	all-*trans* Retinoic acid	SF-1
AR	testosterone	RXR α,β,γ	9-*cis* retinoic acid	COUP-TF
MR	aldosterone	PPAR α,β,γ	prostanoids, fatty acids	TLX
		FXR	farnesoids, bile acids	NURR

Fig. 1a, b The nuclear hormone receptor superfamily. **a** Schematic diagram showing the organization and conserved domains of nuclear hormone receptors. The conserved DNA- and ligand-binding domains are shaded; Activation functions 1 and 2 (AF1 and AF2) are indicated. **b** Some representative examples of members of the nuclear hormone receptor superfamily. The nuclear receptors are subdivided into three types: class I (steroid), class II (non-steroid) and orphan

between the direct repeats dictates NR-binding specificity (Naar et al. 1991; Umesono et al. 1991). In addition to class I and II NRs, a third class of "orphan receptors" has been reported, for which the existence or identity of a ligand has yet to be determined (Mangelsdorf and Evans 1995).

A major breakthrough in our understanding of how NRs regulate gene expression comes from the recent discovery of NR-associated coregulatory factors (reviewed in Aranda and Pascual 2001; Glass and Rosenfeld 2000; McKenna and O'Malley 2002). NR-binding cofactors that enhance transcriptional activation are termed coactivators. The p160/SRC family of proteins is probably the best-characterized of the NR coactivators (Glass and Rosenfeld 2000). Each member of the p160/SRC family has a central NR-interaction domain that contains three copies of a consensus leucine-rich motif, LXXLL (also termed NR box) (Heery et al. 1997). Crystallographic and biochemical studies have revealed that the surface of a single LXXLL motif directly contacts the ligand-activated AF2 domain of NRs, thereby providing a molecular basis for NR-coactivator recruitment (Darimont et al. 1998; Nolte et al. 1998; Shiau et al. 1998). The p160/SRC proteins can also bind histone acetyltransferases (HATs) (Kamei et al. 1996), histone methyltransferases (HMTs) (Chen et al. 1999), and components of the ATP-dependent SNF/SWI chromatin remodeling complex (Belandia et al. 2002) and are thus thought to function by recruiting chromatin-modifying activity to NRs (reviewed in Tsai and Fondell 2004).

In parallel to the identification and functional characterization of NR-coactivators, a distinct set of cofactors that bind NRs (e.g., TR and RAR) in the absence of ligand were also identified. These cofactors were found to confer transcriptional repression, and were therefore termed corepressors (reviewed in Hu and Lazar 2000). Notably, two NR corepressors N-CoR (Horlein et al. 1995) and SMRT (Chen and Evans 1995) associate in vivo with histone deacetylase (HDAC)-complexes and ultimately target this activity to promoter-bound class II NRs in the absence of ligand. A general model of NR-regulated gene expression has thus emerged in which the presence or absence of ligand differentially dictates NR-recruitment of opposite types of chromatin-modifying enzymatic activities to target genes (Glass and Rosenfeld 2000; McKenna and O'Malley 2002).

Adding to the complexity of NR-dependent transcription, the human Mediator complex was first discovered as a multimeric group of proteins that copurifies with TR in the presence of T3 (Fondell et al. 1996). Indeed, the complex was originally termed TRAP for "TR-associated protein" complex. In contrast to the p160/SRC coactivators, the TRAP/Mediator complex possessed no intrinsic histone/chromatin-modifying activity. Nevertheless, the complex significantly enhanced TR-dependent transcription in vitro on non-chromatin templates (Fondell et al. 1996, 1999; Ito and Roeder 2001). This finding suggested that TRAP/Mediator facilitated an activation step distinct from the chromatin-modifying/-remodeling activity of p160/SRC complexes (outlined in more detail below, "Functional role of Mediator complex in transcriptional regulation"). Additional evidence for a more common Mediator coactivator role in NR-signaling pathways came from the isolation of a similar, if not identical complex termed DRIP that associates with VDR and stimulates VDR-dependent transcription in vitro (Rachez et al. 1999). The two complexes were later found to comprise at least 30 subunits (Table 1). Of these, 22 subunits are homologs of yeast proteins found within yeast Mediator, a large 25-subunit complex that directly associates with yeast RNA pol II and is essential for yeast viability (reviewed in Kornberg 2005) (see below, "Evolutionary conservation of Eukaryotic Mediator complexes"). End Grabbed content

Since the initial discovery of Mediator as a coactivator for TR, several laboratories have demonstrated that Mediator directly binds and coactivates transcription for a broad range of both class I (steroidal) and class II (non-steroidal) NRs including ER, AR, GR, RAR, RXR, PPAR, and FXR (Burakov et al. 2002; Ge et al. 2002; Hittelman et al. 1999; Kang et al. 2002; Pineda Torra et al. 2004; Wang et al. 2002; Warnmark et al. 2001; Yuan et al. 1998; Zhu et al. 1997, 1999), as well as the orphan receptors retinoic acid receptor-related orphan receptor (ROR) and hepatocyte nuclear factor 4 (HNF4) (Atkins et al. 1999; Malik et al. 2002). Collectively, these findings implicate the Mediator complex in facilitating an essential transcriptional activation step during NR-regulated gene expression in concert with the chromatin modifying/remodeling activity facilitated by other types of NR-coactivators and -corepressors.

Evolutionary conservation of eukaryotic Mediator complexes

The Mediator complex was originally identified in baker's yeast *Saccharomyces cerevisiae* using both biochemical and genetic methodologies (Gustafsson et al. 1997; Kim et al. 1994; Thompson et al. 1993). The yeast complex was ultimately shown to be composed of 25 subunits (Fig. 2). Most of these subunits can associate, as a group, with the C-terminal domain (CTD) of the largest subunit of yeast RNA pol II (reviewed in Kornberg 2005). Indeed, yeast RNA pol II can be isolated as a holoenzyme containing the Mediator complex. This assemblage is thought to be a regulatory target for gene-specific activators (reviewed in Myer and Young 1998; Myers and Kornberg 2000). Parallel studies further established a role for yeast Mediator in gene-specific repression as well as activation (reviewed in Carlson 1997). Significantly, a conditional mutation of the yeast MED17/Srb4 subunit completely abolishes all RNA pol II transcription (Holstege et al. 1998). This finding thus demonstrated that Mediator is required for the transcription of virtually all protein-encoding genes in yeast.

In humans, a number of highly related complexes sharing conserved subunit homology with yeast Mediator were subsequently identified. The first such complex was TRAP/Mediator, isolated by virtue of its ability to interact with TR in the presence of

Table 1 Eukaryotic Mediator complexes

Unified Nomenclature	S. cerevisiae	D. melanogaster	Homo sapiens				
			TRAP/Mediator	**ARC/DRIP**	**CRSP**	**PC2**	**Others**
MED1	Med1	*TRAP220*	TRAP220	ARC/DRIP205	CRSP200	TRAP220	PBP
MED2	**Med2**						
MED3	**Pgd1/Hrs1/Med3**						
MED4	Med4	Trap36	TRAP36	ARC/DRIP36	ARC/DRIP36	TRAP36	p34
MED5	**Nut1**						
MED6	Med6	Med6	hMed6	ARC/DRIP33	hMed6	hMed6	p32
MED7	Med7	*Med7*	hMed7	ARC/DRIP34	hMed7	hMed7	p36
MED8	Med8	*Arc32*	ARC32	ARC32	ARC32	ARC32	hMed8
MED9	Cse2/Med9	*CG5134*	Med9			(Med25)	
MED10	Nut2/Med10	*Nut2*	hNut2	hMed10	hMed10	hNut2	
MED11	Med11	Med21	HSPC296	HSPC296	HSPC296	HSPC296	
MED12	Srb8	*Kto*	TRAP230	ARC/DRIP240			
MED13	Ssn2/Srb9	*Skd/Pap/Bli*	TRAP240	ARC/DRIP250			
MED14	Rgr1	Trap170	TRAP170	ARC/DRIP150	CRSP150	TRAP170	p110
MED15	Gal11	*Arc105*	PAQ	ARC105	ARC105	PAQ	TIG-1
MED16	Sin4	*Trap95*	TRAP95	DRIP92	DRIP92	TRAP95	p96b
MED17	Srb4	Trap80	TRAP80	ARC/DRIP77	CRSP77	TRAP80	p78
MED18	Srb5	p28/CG14802	p28b			p28b	
MED19	Rox3	*CG5546*	LCMR1	LCMR1	LCMR1	LCMR1	
MED20	Srb2	Trfp	hTRFP	hTRFP	hTRFP	hTRFP	p28a
MED21	Srb7	Trap19	hSrb7	hSrb7	hSrb7	hSrb7	p21
MED22	Srb6	Med24	Surf5	Surf5	Surf5	Surf5	
MED23		***TRAP150β***	**TRAP150β**	**ARC/DRIP130**	**CRSP130**	**TRAP150β**	**hSur2**
MED24		***TRAP100***	**TRAP100**	**ARC/DRIP100**	**CRSP100**	**TRAP100**	
MED25		***Arc92***	**TRAP97**	**DRIP97**	**DRIP97**	**TRAP97**	**ACID1**
MED26		***Arc70***	**CRSP70**		**CRSP70**	**CRSP70**	**p78**
MED27		***Trap37***	**TRAP37**			**TRAP37**	
MED28		**Med23**	**Med28**	**Med28**	**Med28**	**(Med28)**	**Fksg20**
MED29		***Intersex***	**Med29**			**(Med29)**	**Hintersex**
MED30		**Trap25**	**TRAP25**	**TRAP25**	**TRAP25**	**TRAP25**	
MED31	Soh1	Trap18	hSoh1	hSoh1	hSoh1	hSoh1	
CDK8	Srb10	Cdk8	Cdk8	Cdk8			
CycC	Srb11	CycC	CycC	CycC			

Subunits shown in bold indicate proteins which are not evolutionarily conserved between yeast and metazoans. Subunits shown in italics represent proteins that have not yet been identified in biochemically purified complexes.

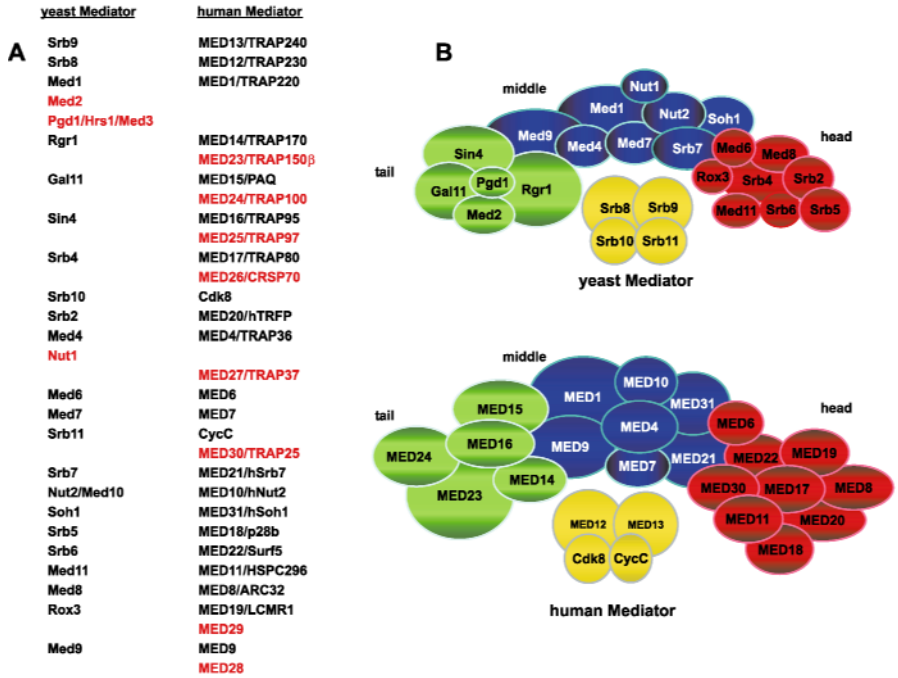

Fig. 2a, b Comparison of the human and yeast Mediator complexes. **a** Subunit comparison of human Mediator and yeast Mediator. Subunits shown in *black* are conserved in both species. Nonconserved subunits specific to either humans or yeast are shown in *red*. **b** Modular organization of yeast Mediator and human Mediator. The topological models for both complexes are based on a number of genetic, biochemical, and structural studies (see text for references). Both complexes are organized in a similar modular structure consisting of a head (*red*), middle (*blue*), and tail (*green*) domain. The Cdk8 module (shown in *yellow*) is variably associated with the core complex, and its precise interaction site remains poorly defined. Recently identified human Mediator subunits whose structural location within the core complex remains uncharacterized are not shown

ligand (Fondell et al. 1996). This was followed by the isolation of other Mediator-like complexes variously termed ARC, DRIP, CRSP, PC2, NAT, and human Mediator (Boyer et al. 1999; Malik et al. 2000; Naar et al. 1999; Rachez et al. 1999; Ryu et al. 1999; Sun et al. 1998). The composition of these independently isolated complexes initially appeared to vary considerably. However, a recent sophisticated proteomic analysis suggests that all human Mediator complexes share at least 30 subunits (Sato et al. 2004), 22 of which are metazoan homologs of *S. cerevisiae* Mediator subunits (Table 1).

In addition to yeast and humans, conserved Mediator subunits have been genetically identified in *Caenorhabditis elegans* and demonstrated to play a critical role in growth and development (Kwon et al. 1999; Singh and Han 1995; Wang et al. 2004a). Furthermore, genetic and biochemical analyses in *Drosophila melanogaster* have revealed a Mediator complex that closely resembles the human complex with regard to size, complexity, and physiological importance (Gim et al. 2001; Park et al. 2001; Table 1). The existence of conserved Mediator complexes from such diverse eukaryotic species has led to a unified nomenclature for eukaryotic Mediator subunits (Table 1, column 1; Bourbon et al. 2004). Of note, humans and other metazoans express eight Mediator subunits that are not conserved in yeast: MED23, MED24, MED25, MED26, MED27, MED28, MED29, and MED30 (Fig. 2; see Ta-

ble 1). Thus, it seems conceivable that these additional subunits in the metazoan complexes likely evolved to facilitate Mediator integration into more complex signaling pathways.

In general, human Mediator complexes can be isolated as two distinct entities. The first is a larger, approximately 2-MDa holocomplex termed TRAP/Mediator or ARC/DRIP. The second is a smaller, approximately 0.7-MDa core complex termed PC2 or CRSP, which is likely a derivative of the larger complex (Malik et al. 2005; Taatjes et al. 2002). The larger TRAP/Mediator and ARC/DRIP complexes contain the subunits MED12, MED13, cyclin C, and cyclin-dependent kinase 8 (Cdk8). By contrast, these four subunits are absent from the smaller PC2 and CRSP complexes which additionally contain the subunit MED26/CRSP70. While the larger TRAP/Mediator and ARC/DRIP complexes have been implicated in both positive and negative transcriptional responses, the smaller PC2 and CRSP smaller complexes appear to be exclusively involved in positive regulatory pathways (Akoulitchev et al. 2000; Fondell et al. 1996; Taatjes et al. 2002; Wang et al. 2004b; Wu et al. 2003; Taatjes et al. 2002).

Electron microscopy and biochemical assays have revealed that both yeast and human Mediator complexes are organized in a similar modular structure comprising a head, middle, and tail domain (Fig. 2; Asturias et al. 1999; Dotson et al. 2000; Guglielmi et al. 2004; Taatjes et al. 2002). The head and tail domains appear to be direct targets for gene-specific activators. For instance, the yeast activator Gal4 can interact directly with subunits MED3 and MED15 in the tail module or MED17 in the head module (reviewed in Boube et al. 2002). Similarly in humans, the transcriptional activators Elk-1 and E1A can directly contact MED23 in the tail module (Stevens et al. 2002), whereas p53 can contact the MED17 subunit in the head module (Ito et al. 1999). Electron microscopy shows that the middle and head modules of yeast Mediator can additionally directly contact yeast RNA pol II (Davis et al. 2002) and that the overall structure of yeast Mediator can change, becoming more extended when it is associated with RNA pol II (Chadick and Asturias 2005). Similar studies with human Mediator show that the complex can adopt different conformations upon binding distinct types of activators (Taatjes et al. 2004; Taatjes and Tjian 2004). Taken together, these studies suggest that the Mediator complex is a dynamic entity and structurally flexible.

In addition to the head, middle, and tail modules, the subunits MED12, MED13, cyclin C, and Cdk8 are thought to constitute a fourth distinct module referred to simply as the Cdk8 module (Fig. 2). The Cdk8 module is variably associated with the core Mediator complex in both yeast and humans, and in yeast can be purified as a separate entity (Borggrefe et al. 2002). Genetic evidence in yeast suggests that the Cdk8 module is involved in negative regulation of gene expression (Carlson 1997; Hengartner et al. 1998; Holstege et al. 1998). In humans, Mediator complexes containing the Cdk8 module have been demonstrated to be transcriptionally inactive in vitro (Taatjes et al. 2002) or implicated in transcriptional repression (Akoulitchev et al. 2000; Mo et al. 2004; Sun et al. 1998) (see below, "Functional role of Mediator complex in transcriptional regulation"). Nonetheless, human Mediator complexes containing the Cdk8 module (e.g., TRAP/Mediator) are clearly recruited to target gene promoters by distinct activators both in vivo and in vitro and suggest that this module may, under certain conditions, play a functional role in some aspect of transcriptional activation prior to dissociating from the core complex (Cantin et al. 2003; Malik et al. 2005; Wang et al. 2004b).

Targeting Mediator to nuclear receptors

The initial observation showing that Mediator copurifies with TR from human cells cultured in T3 suggested that the complex associates with TR and other NRs in a ligand-dependent manner (Fondell et al. 1996). Protein binding assays using purified Mediator have confirmed this notion and showed that TR specifically bound to the 220-kDa subunit (MED1/TRAP220) in the presence of T3 (Yuan et al. 1998). Similarly, association of the Mediator complex with VDR in the presence of ligand was also found to be dependent on MED1/TRAP220 (Rachez et al. 1998). In parallel yeast two-hybrid cloning studies using PPAR as bait, mouse MED1/TRAP220 was identified and shown to bind PPAR, RAR, RXR, and TR in vitro in a ligand-dependent fashion (Zhu et al. 1997). Interestingly, sequence analysis of both the mouse and human MED1/TRAP220 proteins revealed the presence of two centrally located LXXLL signature motifs or NR boxes (Fig. 3a). As noted earlier, NR boxes are commonly found in a number of NR coactivators including the p160/SRC family of proteins (Heery et al. 1997). Given that NR boxes act as direct binding surfaces for ligand-activated AF2 domains of NRs (Darimont et al. 1998; Nolte et al. 1998; Shiau et al. 1998), their presence in MED1/TRAP220 provided a key clue as to how NRs target Mediator.

The presence of two NR boxes (termed NR box 1 and box 2) in the MED1/TRAP220 open reading frame (Fig. 3a) suggested that each motif (or box) might act as an equally potent NR-binding site, or alternatively, each motif might confer different binding affinities for distinct NRs. Several studies have demonstrated the latter. For example, a clear binding preference of class II NRs (e.g., TR, VDR, and PPAR) for NR box 2 is evident when the interactions are studied in the absence of DNA (Rachez et al. 2000; Ren et al. 2000; Yuan et al. 1998). Interestingly, mutagenesis of the amino acid residues immediately flanking NR box 2 revealed a cluster of basic/polar residues N-terminal to the core LXXLL motif (K640, N641, H642) (Fig. 3b) whose presence was shown to be essential for TR, VDR, and PPAR binding (Ren et al. 2000). A similar cluster of basic residues is found N-terminal to NR box 2 in the p160/SRC coactivator GRIP1 (Fig. 3b) and, similar to the situation with MED1/TRAP220, both TR and VDR selectively bind to this NR box (Darimont et al. 1998; Ding et al. 1998). These findings suggest that preferential binding of class II NRs at MED1/TRAP220 NR box 2 is due, at least in part, to conserved basic/polar residues N-terminal to the core LXXLL motif. Indeed, crystallography studies with TR and GRIP1 suggest that these conserved residues electrostatically interact with conserved acidic residues in the AF2 domain of NRs, presumably serving to stabilize the NR-coactivator interaction (Darimont et al. 1998).

When NR-MED1/TRAP220 binding studies are performed using DNA-bound class II NRs heterodimerized with RXR, both NR box 1 and box 2 are required (Rachez et al. 2000; Ren et al. 2000). Indeed, point mutation of either NR box 1 or 2—or deletion mutations changing the conserved spacing between the two NR boxes—significantly disrupts MED1/TRAP220 binding to DNA-bound RXR–TR or RXR–VDR heterodimers (Ren et al. 2000). Furthermore, when binding studies are performed using class II NR heterodimers with opposite DNA-binding polarity (e.g., PPARγ-RXR), a selective requirement for NR box 1 is observed (Yang et al. 2000). Finally, in vitro transcription studies using MED1/TRAP220 point mutants confirm the notion that both NR box 1 and 2 are required for efficient RXR–TR-mediated gene activation (Malik et al. 2004). Taken together, these findings suggest that both NR boxes 1 and 2, properly spaced, are necessary for MED1/TRAP220 to functionally interface with a DNA-bound RXR–NR heterodimer

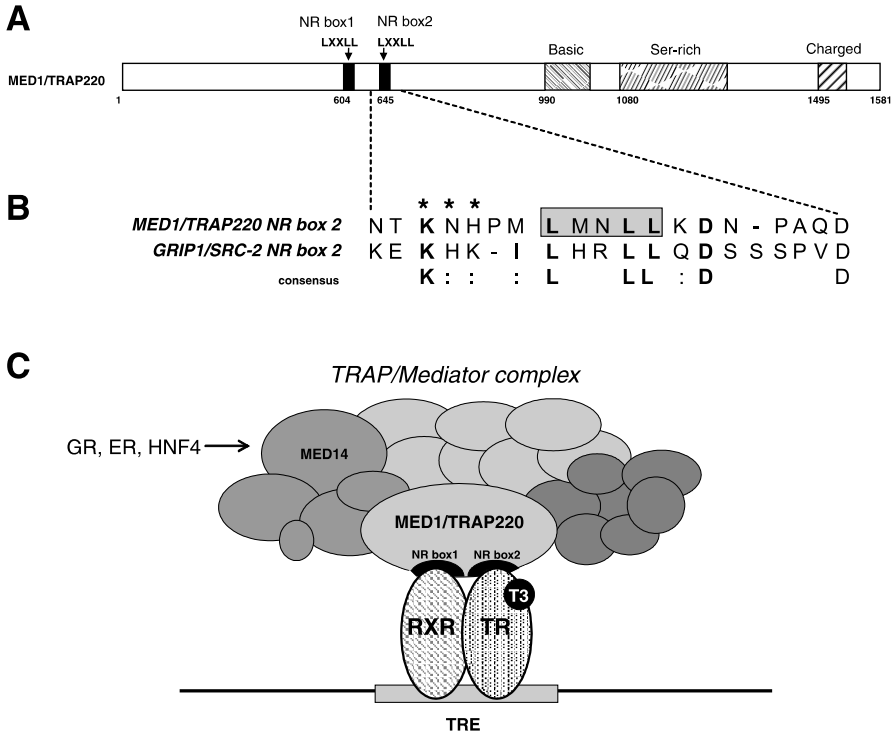

Fig. 3a–c MED1/TRAP220 targets the Mediator complex to NRs. **a** Schematic depiction of the MED1/TRAP220 protein showing the location of the two LXXLL motifs (*black bars*) and regions rich in basic, serine, and charged amino acid residues. **b** Comparison of the core LXXLL motif plus immediate flanking residues of MED1/TRAP220 NR box 2 with the corresponding NR box 2 of the p160/SRC family member GRIP1. Identical consensus residues are shown in *bold*; similar residues are indicated by a *colon* (:). Basic/polar residues flanking the core LXXLL motif of MED1/TRAP220 NR box 2 that are essential for NR binding (Ren et al. 2000) are indicated by an *asterisk*. **c** Model for Mediator binding to class II NRs. In the presence of thyroid hormone (T3), DNA-bound RXR–TR heterodimers simultaneously contact both NR boxes of MED1/TRAP220, with the AF2 domain of RXR contacting NR box 1 and the AF2 domain of TR contacting NR box 2. Additional AF1-dependent interactions between class I and orphan NRs are mediated via the MED14 subunit

and further suggest that NR box 1 might be a specific interaction site for RXR (Coulthard et al. 2003; Ren et al. 2000; Fig. 3c).

In addition to MED1/TRAP220, other Mediator subunits have been implicated as NR-binding targets. For instance, a yeast two-hybrid screen using the N-terminal AF1 domain of GR as bait led to the isolation of MED14 (Hittelman et al. 1999). Given that the C-terminal AF2 domain of GR can still bind MED1/TRAP220 in a ligand-dependent manner, a model has been proposed in which MED14 and MED1/TRAP220 serve as a molecular bridge functionally linking the N- and C-terminal GR activation functions AF1 and AF2. These findings also suggest that the mechanism by which class I (steroid) NRs interact with Mediator may be fundamentally different from that required for class II NRs. Interestingly, a similar interaction model has been proposed for the orphan receptor HNF4, involving a primary AF2-dependent interaction with MED1/TRAP220 and a secondary, presumably AF1-dependent, interaction with MED14 (Malik et al. 2002). A recent report has also implicated MED14 as an interaction target for the ER (Lee et al. 2005), although several other studies have clearly

established MED1/TRAP220 as the primary Mediator-binding target for ER in the presence of ligand (Acevedo et al. 2004; Burakov et al. 2000; Kang et al. 2002; Warnmark et al. 2001; Zhu et al. 1999).

Functional role of Mediator complex in transcriptional regulation

Several lines of evidence support the idea that Mediator functions, at least in part, by binding RNA pol II and facilitating its recruitment to target gene promoters (Fig. 4). First, yeast genetic suppressors of an RNA pol II CTD deletion (termed suppressors of RNA polymerase B or SRBs) were found to be Mediator subunits (Kim et al. 1994; Liao et al. 1995; Myers et al. 1998). Second, yeast Mediator can be purified from cellular extracts tightly associated with RNA pol II (Hengartner et al. 1995; Kim et al. 1994; Thompson et al. 1993) and mutations in the CTD of RNA pol II, or in the Mediator subunits MED17, MED18, or MED20, disrupt recruitment of RNA pol II to a promoter in vitro (Ranish et al. 1999). Similarly, human RNA pol II-Mediator complexes have also been isolated (Malik et al. 2005; Sato et al. 2004) and distinct human Mediator subcomplexes (e.g., CRSP) have been found to directly interact with the CTD of human RNA pol II (Naar et al. 2002). Third, activator-dependent recruitment of human Mediator to a promoter in vitro was found to enhance the subsequent recruitment of RNA pol II in a highly purified reconstituted transcription system (Wu et al. 2003). Fourth, chromatin immunoprecipitation (ChIP) assays (in which the temporal recruitment of RNA pol II and other specific coregulatory factors to native gene promoters can be measured in vivo) show that recruitment of Mediator to ER-target genes occurs concomitantly with, or is followed shortly thereafter by, the recruitment of RNA pol II (Metivier et al. 2003; Shang et al. 2000). These studies thus suggest that Mediator can be recruited to target genes as a preformed RNA pol II-associated holocomplex, or alternatively, as a separate entity that subsequently facilitates the recruitment of RNA pol II.

In addition to RNA pol II recruitment, other studies suggest that Mediator can facilitate the recruitment and binding of the basal transcription factor TFIID at the core promoter. For

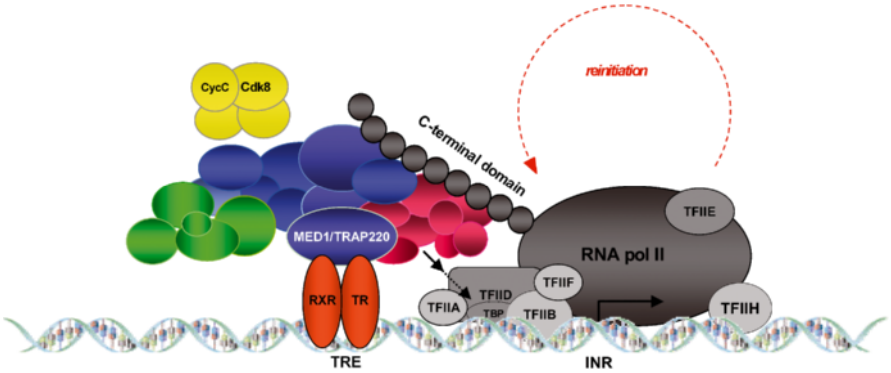

Fig. 4 T3-dependent transcriptional activation via the Mediator complex. RXR–TR heterodimers bound to a T3-response element (*TRE*) bind MED1/TRAP220 in the presence T3 thereby recruiting Mediator to the promoter. Direct interactions between the C-terminal domain of RNA pol II and head (*red*) and middle (*blue*) modules of the Mediator complex are indicated. Mediator-dependent recruitment of the TATA-binding protein (*TBP*) and/or TFIID are indicated by *solid* and *dashed arrows*. A coactivator role for Mediator in transcriptional reinitiation is indicated by a *dashed red circular arrow*. A potential coactivator role for the variably associated Cdk8 module has been suggested by some studies but remains undefined

example, immobilized DNA template assays show that Mediator and TFIID bind cooperatively on test promoters and that high levels of transcription are achieved only after activator-TFIID-Mediator intermediates are formed at the promoter (Johnson et al. 2002). Similarly, other in vitro transcription studies show that Mediator can facilitate promoter recognition by the TATA-binding protein (TBP) component of TFIID in the presence of specific transcriptional activators (Wu et al. 2003; Fig. 4). Important in this regard, this study and others suggest that Mediator can coactivate transcription independently of the TBP-associated factors (TAFs) found within TFIID (Fondell et al. 1999; Wu et al. 2003).

While the precise mechanisms remain undefined, Mediator also appears to be required for transcription initiation steps subsequent to RNA pol II-preinitiation complex recruitment (Malik et al. 2002; Wang et al. 2005). One clue as to how Mediator might facilitate this action comes from studies in yeast in which purified Mediator was shown to stimulate the RNA pol II CTD-kinase activity of the basal transcription factor TFIIH (Kim et al. 1994), an activity that triggers transcriptional initiation. These studies raise the intriguing possibility that TRAP/Mediator remains functionally active at the promoter following the recruitment of RNA pol II and the basal transcription machinery. Along these same lines, Mediator has also been implicated in transcriptional reinitiation. In yeast, mutation of the Mediator subunit MED3 leads to defects in transcriptional reinitiation in vitro (Reeves and Hahn 2003). Similarly, another study using immobilized DNA templates and an in vitro transcription assay showed that following transcriptional initiation, a subset of basal transcription factors (TFII-A, -D, -F, and -H) and yeast Mediator remain at the core promoter, presumably acting as a platform for the subsequent assembly of a reinitiation complex (Yudkovsky et al. 2000). Interestingly, the platform can be stabilized by distinct types of activators, yet not by others. Recently in humans, the formation of a similar reinitiation platform complex containing the Mediator complex was proposed for ER-mediated transcription (Acevedo and Kraus 2003).

Finally, and as alluded to earlier, Mediator has also been implicated in transcriptional repression, primarily via the variably associated Cdk8 module (Figs. 2 and 4). Three possible mechanisms might account for this negative regulation. First, studies in both human and yeast *Schizosaccharomyces pombe* systems suggest that association of the Cdk8 module with the core Mediator complex may occlude interactions with RNA pol II (Naar et al. 2002; Samuelsen et al. 2003). Indeed, in yeast *S. cerevisiae*, the Cdk8 module can phosphorylate the CTD of RNA pol II in vitro (Borggrefe et al. 2002) and the yeast Mediator core complex preferentially interacts with the unphosphorylated form of RNA pol II (Myers et al. 1998). Second, in humans, Cdk8 has been shown to phosphorylate the cyclin H subunit of TFIIH which then inactivates both the CTD kinase activity of TFIIH and its ability to initiate transcription (Akoulitchev et al. 2000). Third, both yeast and mammalian Cdk8 are able to phosphorylate gene-specific transcriptional activators, thus targeting them for ubiquitination and proteasome-based degradation (Chi et al. 2001; Fryer et al. 2004).

Despite these findings, human Mediator complexes containing the Cdk8 module are clearly recruited to target genes in an activator-dependent manner and appear to support transcriptional activation under certain conditions (Fondell et al. 1996; Malik et al. 2005; Rachez et al. 1998; Wang et al. 2004b; Wu et al. 2003). Important in this regard, recent studies in yeast indicate that the presence of the Cdk8 module is essential for Gal4-dependent activation of the *GAL1* gene in vivo (Larschan and Winston 2005). Taken together, these studies demonstrate multiple positive and negative roles for the Mediator complex at target gene promoters that are likely manifested in an activator- and/or gene-specific manner.

Multistep model of nuclear receptor regulated transcription

The packaging of eukaryotic genomic DNA into a higher ordered chromatin structure acts as a barrier to transcription by inhibiting accessibility of RNA pol II and associated factors. This condensed chromatin structure has a major influence on the mechanisms by which NRs activate and repress target gene expression. Therefore, in addition to coactivators or corepressors that directly interface with the basal transcription apparatus (e.g., Mediator), NRs must also recruit coregulatory factors that modify and structurally remodel chromatin. Key examples of such cofactors include HATs, HDACs, HMTs, and the ATP-dependent SNF/SWI chromatin remodeling complex (Belandia and Parker 2003; Tsai and Fondell 2004). As noted earlier, HATs, HMTs, and some components of the SNF/SWI complex can all directly associate with the p160/SRC family of proteins, which in turn directly contacts NRs in a ligand-dependent manner. Hence, p160/SRC cofactors serve as pivotal adaptor molecules in NR signaling pathways (Glass and Rosenfeld 2000; McKenna and O'Malley 2002). Pertinent to this review, the question arises as to how chromatin-modifying/-remodeling cofactors temporally and functionally cooperate with the Mediator complex at specific NR-target genes.

One possible answer comes from MED1/TRAP220 versus p160/SRC competitive binding studies with NRs. Although the binding of both types of cofactors to most NRs is mutually exclusive and competitive, in vitro binding assays and surface plasmon resonance (SPR) analyses revealed a higher T3-dependent TR affinity for p160/SRC cofactors than for MED1/TRAP220 (Moore and Guy 2005; Treuter et al. 1999). Similarly, SPR analyses and electromobility shift competition assays showed a higher estrogen-dependent ER affinity for p160/SRC proteins than for MED1/TRAP220 (Acevedo et al. 2004; Burakov et al. 2002; Warnmark et al. 2001). Taken together, these data suggest that competition between p160/SRC proteins and TRAP220/MED1 for ligand-dependent NR binding might act as a regulatory step in establishing a sequential activation cascade. Consistent with this notion, coimmunoprecipitation studies demonstrated that TR–p160/SRC–HAT complexes assemble in HeLa cells immediately following T3 stimulation, whereas formation of TR–Mediator complexes occurs later (Sharma and Fondell 2000).

The ChIP assay represents a powerful approach for investigating the temporal recruitment kinetics of distinct NR-coregulatory factor complexes to NR target genes in situ. Accordingly, ChIP assays were used to investigate the recruitment of TR–Mediator and TR–p160/SRC–HAT complexes to the T3-responsive genes *Dio1* and *SERCA* in vivo using human HeLa cells and rat GH3, respectively (Sharma and Fondell 2002). Interestingly, these studies showed that following T3 stimulation, TR recruits the different types of coactivator complexes in at least two sequential steps. p160/SRC proteins in association with the HAT p300 are recruited first and rapidly induce histone acetylation at the promoter. Shortly thereafter, TR–Mediator complexes are recruited. Importantly, significant levels of transcription from both the *Dio1* and *SERCA* genes was not observed until after the recruitment of Mediator (Sharma and Fondell 2002). These findings have led to a model of T3-dependent gene activation by TR (Fig. 5). Consistent with these findings, ChIP assays using the human ER(+) breast cancer cell line MCF7 demonstrated a sequential ER recruitment of p160/SRC-HATs followed by Mediator on estrogen-responsive genes in vivo (Burakov et al. 2002; Metivier et al. 2003; Shang et al. 2000). Interestingly, these studies further showed a cycling of the different coactivators on and off the estrogen-regulated promoters, and strikingly, there was an inverse correlation between p160/SRC and MED1/TRAP220 promoter occupancy (Burakov et al. 2002; Metivier et al. 2003). Furthermore, one of the studies clearly implicated

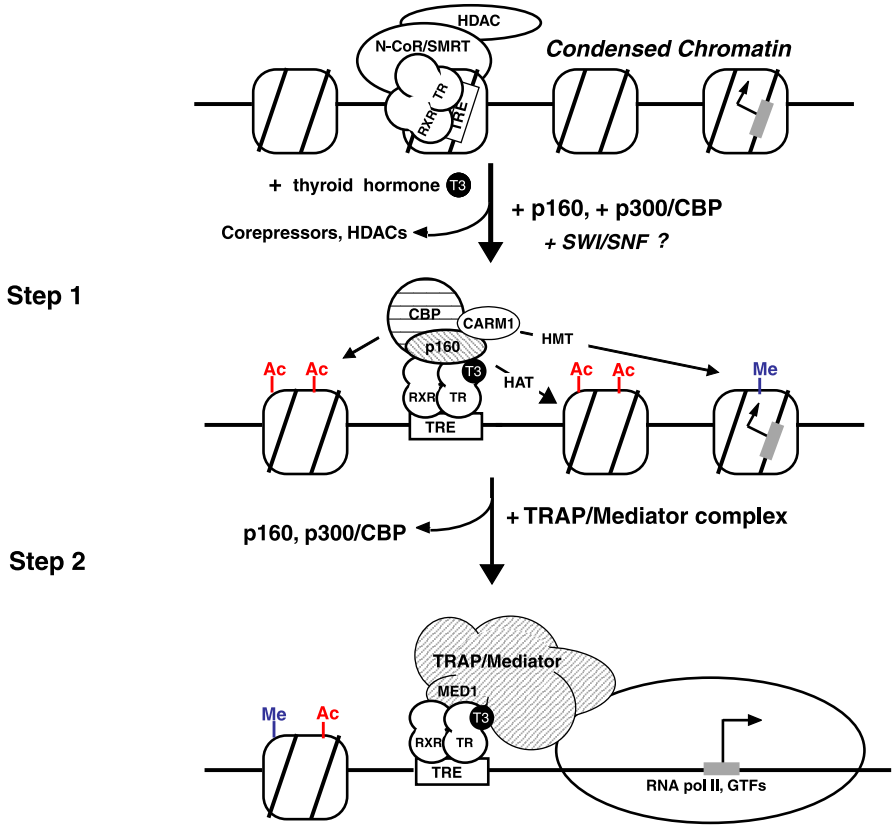

Fig. 5 A multistep model of T3-dependent transcriptional regulation. In the absence of thyroid hormone (T3), RXR–TR heterodimers are constitutively bound to T3-response elements (TREs) in association with the corepressors N-CoR/SMRT and histone deacetylases (HDACs) which lead to condensed chromatin and gene silencing. In the presence of T3, HDACs and corepressors disassociate from RXR–TR, and p160/SRC cofactors are then recruited in association with histone acetyltransferases (HATs; e.g., CBP) and histone methyltransferases (HMTs; e.g., CARM1), which covalently modify promoter proximal histones. This step likely involves the ATP-dependent chromatin-remodeling activity of the SWI/SNF complex. In a temporally subsequent step, the p160/SRC cofactors, HATs, and HMTs disassociate and RXR–TR recruits the Mediator complex, which effectively interfaces with the RNA pol II-basal transcription apparatus to initiate transcription. It is hypothesized that chromatin modifications in the initial step (e.g., acetylation or methylation) may promote the recruitment of TRAP/Mediator to the promoter in the subsequent step (Sharma and Fondell 2002)

SNF/SWI as the first coregulatory complex recruited to an estrogen-responsive promoter following ligand stimulation, where it presumably facilitates the subsequent recruitment of other coactivators (Metivier et al. 2003).

Finally, recent studies suggest that in some instances, Mediator may be poised at specific NR-target genes in the absence of ligand. For example, at the human *RARβ2* gene promoter, which itself is retinoic acid-responsive, Mediator resides pre-bound even in the absence of ligand (Pavri et al. 2005). Upon addition of ligand, corepressor complexes are dismissed and RAR interacts more productively with Mediator in a manner that additionally requires the novel coregulatory factor, poly (ADP-ribose) polymerase-1 (PARP-1). Although the precise mechanism remains ill-defined, it appears that PARP-1 associates with RAR in a ligand-

and promoter-specific manner to trigger the release of the Mediator Cdk8 submodule, which then transcriptionally activates the complex. These findings suggest that other gene-specific cofactors (like PARP-1) may play an important regulatory role in activating the Mediator complex post-promoter recruitment.

The Mediator complex in development and disease

Given the essential role played by the Mediator complex in regulating transcription from eukaryotic protein-encoding genes, a recent focus has been directed toward understanding its physiological importance in mammalian development and disease-related processes. Below we summarize a number of recent studies examining the pathophysiological role of Mediator in mammals with an emphasis on the MED1/TRAP220 subunit and NR signaling pathways.

Genetic ablation of the MED1/TRAP220 subunit in mice results in embryonic lethality at around day 11. The causes of embryonic death are manifold and include hepatic necrosis, defects in hematopoiesis, hypoplasia of the ventricular myocardium, impaired neuronal development, and defects in the development of the placental vasculature (Crawford et al. 2002; Ito et al. 2000; Landles et al. 2003; Zhu et al. 2000). Intriguingly, the *MED1/TRAP220*$^{-/-}$ null embryos exhibit severe retarded cell growth (Ito et al. 2000; Zhu et al. 2000), whereas primary mouse embryonic fibroblasts (MEFs) isolated prior to embryonic death further display impaired cell-cycle progression (Ito et al. 2000). These findings thus suggest that, in addition to mediating embryonic development, MED1/TRAP220 may also play a key coregulatory role in facilitating fundamental mitotic cellular growth. Importantly, TR- and PPAR-dependent gene expression is markedly attenuated in MEFs derived from *MED1/TRAP220*$^{-/-}$ null mutants, but can be restored on transfection with ectopic MED1/TRAP220 (Ito et al. 2000; Zhu et al. 2000). These data thus confirm that MED1/TRAP220 acts as a major physiological coactivator for NRs.

While the genetic ablation studies clearly demonstrated a role for MED1/TRAP220 during embryonic development, a targeted *Cre-loxP* strategy was used to selectively inhibit MED1/TRAP220 expression in the postnatal mouse liver (Jia et al. 2004). Conditional deletion of MED1/TRAP220 in liver parenchymal cells resulted in abrogation of PPARα ligand-induced peroxisome proliferation and liver cell proliferation, as well as the induction of PPARα-regulated hepatic gene expression (Jia et al. 2004). In essence, these findings show that the absence of MED1/TRAP220 in hepatocytes in vivo mimics the absence of PPARα, thus demonstrating that MED1/TRAP220 is essential for PPARα-mediated gene expression in the liver. Whereas PPARα is crucial for liver function, PPARγ is a key regulator of transcriptional pathways essential for adipogenesis (Lowell 1999). Interestingly, it was found that *MED1/TRAP220*$^{-/-}$ null MEFs are refractory for PPARγ-stimulated adipogenesis, but the defect could be restored upon expression of exogenous MED1/TRAP220 (Ge et al. 2002). Taken together, these findings indicate that MED1/TRAP220 acts in vivo (via the Mediator complex) as a PPARα- and PPARγ-specific coactivator in liver and adipose tissue.

A conditional *Cre-loxP* strategy was also used to examine the role of MED1/TRAP220 during mammary gland development in mice (Jia et al. 2005). MED1/TRAP220-deficient mammary glands exhibited retarded ductal elongation during puberty and decreased alveolar density during pregnancy and lactation. Furthermore, the MED1/TRAP220-deficient mammary glands could not produce milk and failed to respond proliferatively to estrogen and progesterone treatment (Jia et al. 2005). Therefore, and similar to the conditional

MED1/TRAP220 ablation in the liver, the absence of TRAP220/MED1 in mammary tissue mimics the absence of ER and PR, thus demonstrating once again that TRAP220/MED1 is essential for NR-mediated gene expression in mammary glands. Important in this regard, MED1/TRAP220 is overexpressed in ER-positive primary human breast cancers and breast cancer cell lines and has been thus been proposed to play an important role in breast cancer progression (Zhu et al. 1999).

Paget's disease is a chronic bone disorder that is characterized by increased osteoclastic bone resorption, excessive bone formation, and hypersensitivity to physiological vitamin D3 concentrations (Hosking 1981). Interestingly, expression of MED1/TRAP220, a direct coactivator for VDR, is amplified in osteoclast precursors in patients with Paget's disease (Kurihara et al. 2004). These findings thus suggest that the pathophysiology underlying this bone disorder may involve increased vitamin D3-dependent transcription via increased levels of VDR coactivators like Mediator.

Immunohistochemistry studies show that MED1/TRAP220 is differentially expressed in the neurons of the granular layer of the cerebellar cortex, piriform cortex, and hippocampus in brains of developing mice and rats (Galeeva et al. 2002). These findings support the notion that MED1/TRAP220 plays an important role in the growth and differentiation of the central nervous system and may have specific functions in certain areas of adult brain. Consistent with an important role for the Mediator complex in the development of the central nervous system, mutations in the MED12 subunit correlate with human schizophrenia, dementia, hypothyroidism, and mental retardation (Philibert et al. 2001). Furthermore, a recently identified component of the complex, MED13L, was found to be mutated in patients with both mental retardation and congenital heart defects (Muncke et al. 2003). Interestingly, the *Drosophila* homologs of MED12 and MED13, *blind spot* and *kohtalo*, are required for proper eye-antennal development (Treisman 2001). Similarly, several labs have demonstrated that the *C. elegans* homologs of MED12 and MED13 are essential for neurodifferentiation, development, and embryogenesis (Moghal and Sternberg 2003; Wang et al. 2004a). Taken together, these studies suggest that Mediator plays an important and highly conserved role during metazoan development of the central nervous system.

Future directions

The Mediator complex clearly plays a central role in regulating NR-dependent transcription from mammalian protein-encoding genes. Nevertheless many questions must still be answered before we can fundamentally understand how the complex works and how its activities are controlled. In this regard, a better understanding of the functional and enzymatic activity of the Cdk8 module, its nuclear targets, and how its association with the core Mediator complex is regulated will be key to appreciating how the Mediator complex activates and/or represses messenger RNA synthesis. The mechanisms by which regulated chromatin remodeling and histone modifications dictate Mediator recruitment to specific target genes also remain poorly defined. Future studies addressing these issues will be crucial in terms of defining more precisely the specific temporal steps of transcriptional initiation and reinitiation facilitated by the Mediator complex. Another major question concerning Mediator is how specific cellular signal transduction pathways influence the overall functional activity of the complex. It is hypothesized that extracellular growth factors or signals likely trigger specific posttranslational modifications (e.g., phosphorylation, acetylation, methylation) of distinct Mediator subunits that ultimately regulate the functional activity of the complex and

the expression of specific target genes. Finally, and in light of the myriad of recently identified mammalian Mediator subunits, a major challenge ahead will be sorting out the specific functional roles of these various polypeptides and investigating how they interact with other Mediator subunits as well as other types of transcriptional coregulatory factors.

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An update on acid secretion

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Abstract Gastric acid secretion is a complex process that requires hormonal, neuronal, or calcium-sensing receptor activation for insertion of pumps into the apical surface of the parietal cell. Activation of any or all these pathways causes the parietal cell to secrete concentrated acid with a pH at or close to 1. This acidic fluid combines with enzymes that are secreted from neighbouring chief cells and passes out of the gland up through a mucous gel layer covering the surface of the stomach producing a final intragastric pH of less than 4 during the active phase of acid secretion. Defects in either the mucosal barrier or in the regulatory mechanisms that modulate the secretory pathways will result in erosion of the barrier and ulcerations of the stomach or esophagus. The entire process of acid secretion relies on activation of the catalytic cycle of the gastric H^+,K^+-ATPase, resulting in the secretion of acid into the parietal cell canaliculus, with K^+ being the important and rate-limiting ion in this activation process. In addition to K^+ as a rate limiter for acid production, Cl^- secretion via an apical channel must also occur. In this review we present a discussion of the mechanics of acid secretion and a discussion of recently identified transporter proteins and receptors. Included is a discussion of some of the recent candidates for the apical K^+ recycling channel, as well as two recently identified apical proteins (NHE-3, PAT-1), and the newly characterized calcium-sensing receptor (CaSR). We hope that this review will give additional insight into the complex process of acid secretion.

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Introduction

Gastric acid plays a primary role in digestion as well as in the sterilization of food and water. Gastric juice contains the most concentrated physiological acid solution (pH~1) as a result of H^+ and Cl^- ion secretion [hydrochloric acid (HCl) production] by parietal cells in the oxyntic mucosa of the stomach. The combined output of the parietal cells leads to the secretion of 1–2 l of HCl at a concentration of 150–160 mmol/l into the interior of the stomach. In order to facilitate the production of acid, the parietal cell relies on the generation of a high concentration of H^+ ions that are transported into the lumen of the gland. This process is facilitated by activation of the gastric H^+,K^+-ATPase, which translocates to the apical pole of the parietal cell. K^+ as well as ATP hydrolysis and Cl^- all play critical roles in the activation of gastric H^+,K^+-ATPase and are essential for the functioning of the enzyme (Reenstra and Forte 1990).

This review will examine the classical proteins that have been linked to acid secretion as well as some recently identified proteins that may modulate gastric acid secretion, in addition we discuss the known secretagogues, and their receptors including a new receptor, which upon stimulation can lead to acid secretion.

The gastric H^+,K^+-ATPase

During the secretory phase of acid secretion, the gastric H^+,K^+-ATPase is located in the apical membrane or pole of the parietal cell and actively transports H^+ into the parietal cell canaliculus in exchange for K^+ at the expense of one ATP molecule per exchange. The cations exchange in a 1:1 ratio thereby maintaining electroneutrality. This enzyme is a member of the P-ATPase family, which includes: Na^+,K^+-ATPase, Ca^{2+}-ATPase and colonic H^+,K^+-ATPase (Maclennan et al. 1985) (Shull et al. 1985; Crowson and Shull 1992). The gastric H^+,K^+-ATPase shares many features, including structure and enzymatic identity, with other members of the family (Rabon et al. 1982; Malinowska et al. 1988; Mendlein et al. 1990; Hersey and Sachs 1995; Munson et al. 2000; Vagin et al. 2004). A common feature of the P-ATPase family is that energy necessary for the translocation of ions is provided by ATP.

Due to the amount of energy released on hydrolysis of an ATP molecule, the concentration gradient that can be created by a P-ATPase enzyme is effectively limited. In this same regard the transport of ions is also constrained if ionic exchange results in the generation of a charge. As the translocation of H^+ and K^+ is electroneutral, the gastric H^+,K^+-ATPase can achieve an approximate 3–4 million-fold ion concentration gradient (difference in H^+ concentration between plasma and parietal cell canaliculus) that is among the highest observed in the mammalian body (Mangeat et al. 1990; Crothers Jr. et al. 1995; Thangarajah et al. 2002; Yao and Forte 2003).

To generate such a concentrated acid solution during the active process of acid secretion it is also necessary to move other ions. In the classical model of gastric acid secretion, it has been proposed that for each H^+ ion transported into the canaliculus by the H^+,K^+-ATPase, the basolateral Cl^-/HCO_3^- exchanger would deliver an HCO_3^- molecule into the plasma and a Cl^- ion into the cytosol (Fig. 1). This exchange would allow for sufficient Cl^- ions to enter the cell replacing the Cl^- ions released from the cell during secretion into the canaliculus. This Cl^- exit step is thought to occur via Cl^- channel(s) in the apical membrane of the parietal cell, and there is a good possibility that more that one type of channel is involved,

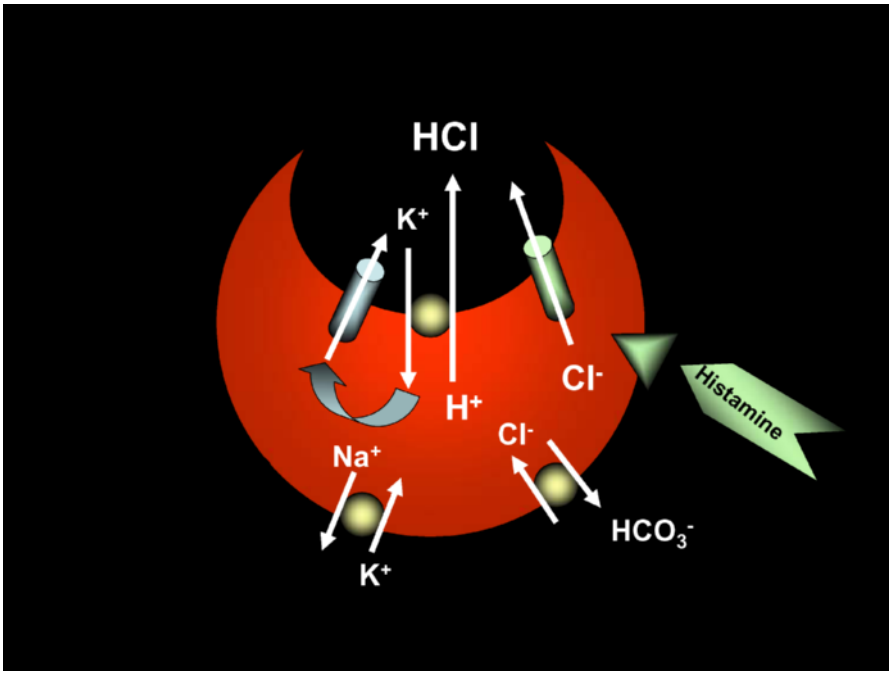

Fig. 1 A simplified model for the secretion of gastric acid by the parietal cell

as is more than likely the case with K^+ channels, based on recent findings (Grahammer et al. 2001; Forte 2004). For Cl^- there has been little direct experimental evidence, and only a ClC-2 channel (Malinowska et al. 1995) has been suggested to play a role in acid secretion. It is essential to have active Cl^- secretion into the lumen of the gland to act as the counter ion for both the flux of K^+ ions and also to balance the charge during H^+ secretion and thereby ensure electroneutral HCl secretion.

Similar to most other cells in the body, the levels of K^+ in the cytosol are higher than in the plasma. The higher intracellular levels of K^+ are dependent on the activity of the Na^+,K^+-ATPase (see Fig. 1). This enzyme is located on the basolateral membrane of the cell where it exchanges intracellular Na^+ for extracellular K^+. The levels of K^+ within the cell are also regulated by K^+ channels that allow ion movement across the basolateral membrane. These channels have a particularly important role in generating negative cell membrane potential.

Gastric H^+,K^+-ATPase activation by K^+

During the resting/non-secretory state, parietal cell H^+,K^+-ATPase is situated along the tubulovesicular elements within the cell (Smolka et al. 1983). In this dormant phase the concentration of K^+ in the tubulovesicular elements is low and the vesicular membranes are impermeable to K^+. As a result, the enzyme is incapable of being activated and transporting H^+ ions (Reenstra and Forte 1990). During stimulation by neuronal, hormonal, or the recently identified CaSR, a morphological change ensues which causes the tubulovesicular elements to fuse with the apical membrane of the cell. During this phase of vesicular fusion the H^+,K^+-ATPase does not appear to undergo any chemical modifications, rather, as a result

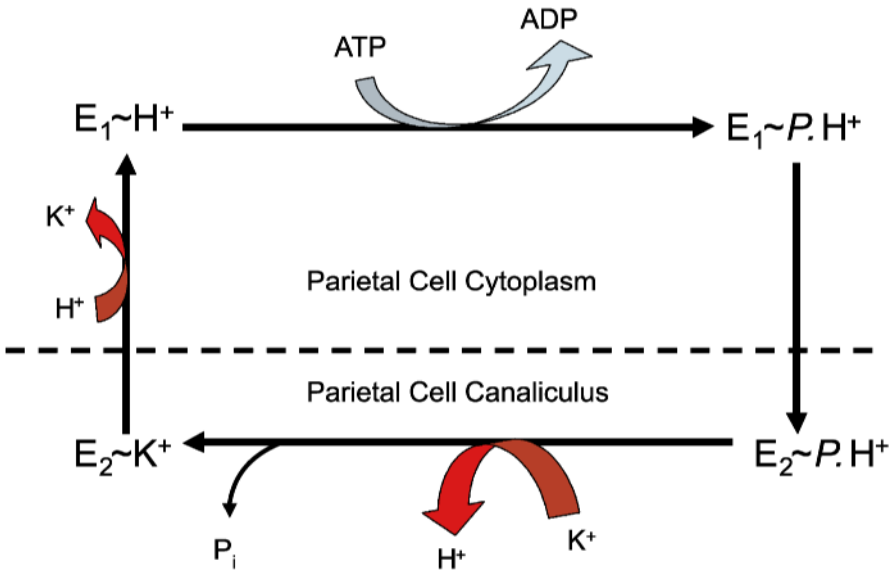

Fig. 2 Post–Albers catalytic cycle of gastric H^+, K^+-ATPase

of the membrane fusion events, the enzyme is now exposed to K^+-containing luminal fluid, and can thus start the active exchange of H^+ for K^+.

To accomplish the exchange of ions, there are several conformational changes that need to occur in the three-dimensional structure of the H^+,K^+-ATPase (Fig. 2). By using the Post–Albers model for the Na-K ATPase, a functional model has been developed to demonstrate the various conformational states that the protein goes to when exposed to increasing levels of ATP. These conformational changes can be summated into two important conformational states that the protein exists in: (1) state 1, which is referred to as E1, in which the ion-binding site faces the parietal cell cytoplasm and has a high affinity for H^+ and low affinity for K^+; (2) state 2 or E_2, where the ion-binding site faces the extracellular canaliculus and has a low affinity for H^+ and high affinity for K^+. It has been postulated that the shape of the K^+ binding site or the path through which K^+ can access the binding site is different for the E_1 and E_2 forms (Vagin et al. 2003). This model would help to elucidate the relative affinity of the two forms for K^+.

While in the E_1 form, the enzyme takes up H^+ and converts to the E_2 form by hydrolysing ATP (see Fig. 2). In addition to providing energy for the shift between these conformational states, ATP hydrolysis also results in the phosphorylation of the enzyme (typically referred to as E_2~P). Transformation to the E_2 form results in the translocation of H^+ from the parietal cell membrane into the secretory canaliculus. As a result, the phosphorylated E_2 form binds K^+ that is required for the dephosphorylation of the H^+,K^+-ATPase.

During this cycling of the enzyme, K^+ becomes temporarily occluded within the transmembrane segments and as a result prevents the cations from having free access to the cytoplasm or canaliculus. While in this phase of the cycle, the cation causes dephosphorylation of the H^+,K^+-ATPase (Rabon et al. 1982). The mechanism responsible for this process has not been fully elucidated. It is, however, likely that K^+ does not stimulate dephosphorylation of the phosphorylated intermediate directly, but rather acts by neutralizing the inhibitory effect

of a negative charge in the membrane (Swarts et al. 1998). Following dephosphorylation, the enzyme returns to the E1 form and releases K^+ into the cell cytoplasm.

The actual amount of H^+ and K^+ that is exchanged during a single cycle remains controversial as some investigators have reported that one H^+ and one K^+ are exchanged for each ATP hydrolysed, while others have found that there is reciprocal exchange of two pairs of ions per ATP hydrolysis(Rabon et al. 1982; Skrabanja et al. 1984). The case for a one H^+ swapped for one K^+ ion has been strengthened by recent modelling work which demonstrated that the gastric H^+,K^+-ATPase has a single K^+ binding site (Koenderink et al. 2004). Additional evidence for the 1:1 exchange is shown in other P-type ATPases, such as yeast and plant H^+-ATPase (Bukrinsky et al. 2001). Moreover, it has been proposed that one single binding site could more easily explain the ability of the H^+,K^+-ATPase to transport H^+ against a high concentration gradient (Koenderink et al. 2004).

The K^+ binding site in gastric H^+,K^+-ATPase

A great deal of work has been focused on elucidating the identity of the K^+ binding site (or sites). To date, potential sites have been identified within the transmembrane segments M4, M5, M6 and possibly M8 of the α-subunit of the enzyme (Munson et al. 2000; Swarts et al. 1996; Vagin et al. 2001; Asano et al. 1996). Due to the physiological role and location of K^+ (i.e. in the parietal cell canaliculus), it would suggest that the site is located in the direction of the luminal face of the membrane domains (Munson et al. 2000). When K^+ occupies this binding site, it seems to affect the conformation of a large intracellular loop in which phosphorylation occurs(Swarts et al. 1998). Moreover, K^+ has been shown to be required for stabilization of a tight loop or 'hairpin' between M5 and M6 (Gatto et al. 1999). This hairpin appears to have a direct link with the phosphorylation domain on the intracellular loop containing the ATP binding site, suggesting that it is involved in coupling ATP hydrolysis with cation transport (Gatto et al. 1999).

As discussed earlier, recent work indicates that there is one high-affinity cation binding site in the gastric H^+,K^+-ATPase (Koenderink et al. 2004). This K^+ binding site is formed by amino acids from M4, M5 and M6, with the K^+ ion being held in place by six oxygen atoms provided by these domains (Koenderink et al. 2004). Within the pocket (at residue 820), there is a negative charge that is thought to be important for enzyme function (Koenderink et al. 2004)

This model of the likely structure of the K^+ site and its interaction with the phosphorylation domain has promoted a theory on how K^+ activates the enzyme. This model suggests that the negative charge in the ion-binding pocket may exert an indirect inhibitory effect on the phosphorylated intermediate form of the enzyme and thereby prevent its hydrolysis(Swarts et al. 1998). When the cation-binding pocket is occupied by K^+, the negative charge is neutralized. This loss of charge causes a signal to be transferred to the nearby phosphorylation domain of the enzyme, possibly via a lysine amino acid residue, resulting in enzyme dephosphorylation (De Pont et al. 2003) causing the subsequent translocation of K^+ from the parietal cell canaliculus to the cytoplasm.

K^+ selectivity of the H^+,K^+-ATPase

The cation selectivity of the enzyme appears to be generated through its interactions with the residues of the transmembrane segments of the α-subunit and the flanking loops that connect

these transmembrane domains (Mense et al. 2002). The degree of K^+ affinity, along with ATPase activity of the gastric H^+,K^+-ATPase, also appears influenced by a salt bridge from M5 to M6 that exists only when the enzyme is in the E_2 form (Koenderink et al. 2004). What appears to be important is that this salt bridge only allows space for a single K^+ binding site, thereby preventing the formation of another K^+ binding site within the enzyme (Koenderink et al. 2004).

The β-subunit has also been implicated in determining K^+ affinity of gastric H^+,K^+-ATPase (Koenderink et al. 1999; Hermsen et al. 2000), as shown in a recent study comparing the pig and rat gastric H^+,K^+-ATPase. The different K^+ affinity of the enzymes from the two species was modulated by both the lipid matrix in which the enzymes were embedded and the identity of the β-subunit (Hermsen et al. 2000).

Role of K^+ channels in K^+ recycling

At resting (i.e. unstimulated, basal) levels of parietal cell activity, gastric juice consists mainly of NaCl with only small amounts of K^+ and H^+. Upon stimulation of the parietal cell there is a sharp drop in pH. When the pH falls to approximately 1 during stimulation, the canaliculus of the parietal cell will contain 150–160 mmol/l HCl. Given the initial low concentration of K^+ ions in the unstimulated state, achieving such a low pH would appear to be difficult through a 1:1 exchange of H^+ for K^+. However, parietal cell stimulation actually elevates the K^+ concentration (measured as KCl) in the gastric milieu (to 10–20 mmol/l KCl). However, even at these levels there would be rapid K^+ depletion unless there was a mechanism for replenishing K^+ levels in the parietal cell canaliculus.

The source therefore of K^+ ions that are exchanged with H^+ by the H^+,K^+-ATPase is the cytosol of the parietal cell, this equilibrium is thought to occur via the Na^+,K^+-ATPase enzyme on the basolateral membrane which accumulates K^+ into the parietal cell in exchange for Na^+. Recently there has been evidence for a NaK2Cl transporter on the basolateral membrane of the cell that would potentially provide both K and Cl for acid secretion (McDaniel and Lytle 1999; McDaniel et al. 2005). This transporter would, however, be linked to the activity of the apical Cl and K channels that are active during acid secretion (McDaniel and Lytle 99)

There have been a variety of candidates to act as an apical K^+ recycling channel, and to date three different types of K^+ channels that may contribute to K^+ recycling, and which have been postulated to be at the apical membrane of the parietal cell, have been investigated in detail.

One of these candidates is the K^+ channel KCNQ1 (formerly known as KvLQT1) which was found to co-localize with gastric H^+,K^+-ATPase and to be abundantly expressed in human and mouse gastric mucosa (Dedek and Waldegger 2001; Grahammer et al. 2001). Using an expression system and an electrophysiological assessment of the KCNQ1 channel it was confirmed that it still had sustained activity at low pH (Dedek and Waldegger 2001; Grahammer et al. 2001). An essential property of any of the apical ion channels involved in acid secretion is their ability to maintain a high open probability when faced with a low pH on the extracellular domain, which occurs during the secretory phase of acid secretion. There is evidence that the subunit KCNE2 (and possibly KCNE3) appears to co-assemble with KCNQ1 to form a functional version of the K^+ channel in the apical membrane of parietal cells (Grahammer et al. 2001). It is postulated that this subunit is thought to determine the

voltage dependence of KCNQ1 and its activation in response to extracellular acidification (Grahammer et al. 2001).

For these reasons as well as recent gene chip identification and amplification of the KCNQ1 protein (Lambrecht et al. 2005), it has been proposed as an important K^+ channel in the apical membrane. Additional evidence of its role in gastric acid secretion came from inhibition studies using the 'specific' KCNQ1 channel inhibitor, chromanol 293B (Grahammer et al. 2001). However, it has since been suggested that chromanol 293B may have an alternative, unidentified target in the parietal cell (Malinowska et al. 2004). In addition there have been conflicting reports as to suppression of acid secretion in knock-out mice, with both positive and negative effects on acid secretion being reported (Lee et al. 2000).

In addition to the KCNQ1 channel, several members of another type of K^+ channel family, the inward rectifying K^+ (Kir) family, have been shown to be expressed in rat gastric mucosa (Fujita et al. 2002). To date, the following Kir channels were detected and they include: Kir4.1, 4.2 and 7.1, although only Kir4.1 and Kir 2.1 have been found in parietal cells (Malinowska et al. 2004).

An additional member of the Kir family may also be involved in gastric acid secretion (Malinowska et al. 2004). In rabbit gastric mucosa, high levels of Kir2.1 were detected along with lower levels of Kir4.1 and 7.1. Kir2.1 was expressed in parietal cells from rabbit gastric mucosa and appeared to co-localize with H^+,K^+-ATPase and ClC-2 Cl^- channels. These K^+ channels were more likely to be open (i.e. allow K^+ transit) when obtained from stimulated stomachs than from resting stomachs. Similarly, a reduction in pH also tended to increase the likelihood of channel openings, which suggests that these channels are regulated in a similar fashion to ClC-2 Cl^- channels. As with KCNQ1 it is not possible to ascribe K^+ conductance in the parietal cell to Kir2.1 alone, since the electrophysiological properties were studied in rabbit gastric vesicles. As with the Kir4.1 channel, the Kir2.1 channel associates with four subunits to form a functional K^+ channel.

Studies thus far have produced evidence for a variety of potassium channels (i.e. KCNQ1, Kir2.1 and Kir4.1), albeit in a variety of different species, in the apical membrane of the parietal cell. All three of the channels described have properties that would be consistent with a K^+ recycling channel. However, it remains uncertain which of these channels, if any, plays the major role in K^+ efflux. As with the Cl^- channel(s), a complete understanding of the K^+ channel(s) involvement has yet to be attained. Additional studies in both native tissues and in transgenic animals may allow a more definitive answer. In addition to the candidates mentioned above as the apical recycling channel(s), a variety of K^+ channels have also been identified on the basolateral membrane of parietal cells, each with distinctive properties (Supplisson et al. 1991). For this reason it is not unreasonable to assume that more than one type of K^+ channel in the apical membrane of the parietal cell may be involved in recycling the cation. In addition, as previously noted, alongside this potential diversity of K^+ channels, different subunits may exist in a single cell, which may affect the properties of the channels (Raap et al. 2002; Wulfsen et al. 2000) This observation raises the possibility of a variety of functional channels with subtly different electrophysiological properties, thereby making elucidation of the relative contribution of different K^+ channels extremely difficult. From the evidence presented there are positive indications that more than one channel protein localizes to the apical region of the gland; as a result, the elimination of one channel could lead to the up-regulation of an alternative channel. This also may be the reason that knock-out mice have not led to a definitive answer as to which channel is the apical recycling channel.

Caution must be applied in attempting to extrapolate the findings in animal studies to man. Until detailed studies are carried out in humans to determine the identity and com-

position of K^+ channels, it will be impossible to determine which channel or channels are important in apical membrane K^+ flux. It also remains to be ascertained whether these channels are constitutively active or are regulated upon cell activation.

K^+ as a target for stopping gastric acid production

As shown in the previous sections of this review, K^+ plays an essential role in the production of gastric acid and therefore makes it a logical potential target for therapeutic intervention. If the K^+ channel(s) responsible for the flow of K^+ ions across the parietal cell apical membrane are inhibited, then gastric H^+,K^+-ATPase will cease to function. An alternative pharmacological approach is to compete with K^+ at the level of the gastric H^+,K^+-ATPase.

K^+ channel blockers

K^+ channel(s) in the apical membrane of the parietal cell represent a target for pharmacological modulation. Recent studies showing the inhibition of gastric acid secretion by exposure to the 'specific' KCNQ1 K^+ channel blocker, chromanol 293B, indicate the potential of such an approach (Grahammer et al. 2001). However, even if a K^+ channel blocker did prevent H^+,K^+-ATPase activity, other challenges hinder the development of a therapeutic K^+ channel blocker: first and foremost, the identity of the channel(s) involved in K^+ recycling in the parietal cell will require further investigation. From recent data presented in this review, and some additional yet unpublished observations, there is an increased likelihood that more than one channel is involved in cation flux (which would then require either several blockers or a drug that could inhibit a variety of channels). Without selectively blocking these additional channels one can speculate that the remaining channels could transport sufficient K^+ to allow the H^+,K^+-ATPase to continue to function. An additional problem facing this approach is the fact that many of the identified gastric K^+ channels can also be found in a variety of tissues [e.g. Kir4.1 is found on brain astrocytes (Higashi et al. 2001) as well as in the apical membrane of parietal cells]. Due to this multi-organ distribution for the channel proteins identified to date, the development of a specific compound or compounds will require the generation of agents that are organ specific, which may be an almost impossible task given the degree of cross-tissue homology that K^+ channel proteins exhibit.

Potassium-competitive acid blockers

In order to distinguish potassium-competitive acid blockers (P-CABs) from proton pump inhibitors (PPIs), a new anachronism has been employed: P-CABs, since this group of compounds competes for the K^+ binding site on the H^+,K^+-ATPase. This class of mechanistically similar developmental compounds was identified as a potential therapeutic option for gastro-esophageal reflux disease and other acid-related disorders(Vakil 2004). Members of this class inhibit gastric H^+,K^+-ATPase by binding ionically to the enzyme and thus preventing proton extrusion and activation by the K^+ cation. It is probable that P-CABs bind at or near the K^+ binding site and so prevent access of the cation to the site.

The oldest member of this group that exemplifies the mode of action is SCH28080. This agent could successfully inhibit gastric acid production in healthy volunteers (Ene et al. 1982) and although clinical development was not continued, the compound has been used extensively to explore the mechanisms of inhibition of gastric H^+,K^+-ATPase.

The large molecular size of SCH28080 compared with K^+ ions suggests that the ion-binding site and inhibitor-binding site are not identical. Furthermore, a mutational analysis of the gastric H^+,K^+-ATPase suggests that there are separate binding sites for SCH28080 and K^+. Examples of this point have been shown by mutations of several amino acid residues in the membrane domains that reduced the affinity for SCH28080 but had no effect on K^+ affinity (Lambrecht et al. 2000; Asano et al. 2004). A mutational analysis also demonstrates that the binding site of SCH28080 appears closer to the luminal surface of the parietal cell than the ion-binding site (Vagin et al. 2003).

It has been proposed that SCH28080 gains access to its binding site and competes with K^+ when the gastric H^+,K^+-ATPase is in the phosphorylated E_2 form (Keeling et al. 1989; Mendlein and Sachs 1990). P-CAB binding to the H^+,K^+-ATPase stabilizes the enzyme in the E_2 conformation and, thereby, prevents movement of H^+ ions into the parietal cell canaliculus. Studies using mutational data suggest that SCH28080 binds near the loop between M5 and M6, and at the luminal end of M6, about two helical turns away from the ion-binding site (Vagin et al. 2003). When examined using homology modelling, it has been suggested that SCH28080 interacts with residues in the M1 to M6 domains (Yan et al. 2004), and, more specifically, SCH28080 (and another P-CAB, SPI-447) docks in a cavity formed by the M1, M4, M5, M6 and M8 transmembrane segments and by loops formed by M5/M6, M7/M8 and M9/M10 (Keeling et al. 1991). The P-CAB molecule is unable to occupy its binding pocket when the enzyme is in the E_1 form due to rearrangement of the loop between M3 and M4, which alters the shape of the P-CAB binding cavity (Asano et al. 2004).

Studies employing animal models as well as early clinical studies have demonstrated that P-CABs appear highly selective for gastric H^+,K^+-ATPase and inhibit gastric acid secretion with a fast onset of action (Keeling et al. 1989; Tsukimi et al. 2000). One interesting observation is that the gastric isoform of the H^+,K^+-ATPase has only been identified in two organs: the stomach and the kidney. Drugs that are targeted directly at the H^+,K^+-ATPase have shown no adverse effects on renal function in either animal models or in humans following prolonged use.

Cl^- entry from the basolateral membrane

The pathway for Cl^- entry into the parietal cell has always been thought to occur via activation of a Cl/HCO_3 exchange pathway located on the basolateral membrane of the parietal cell (Paradiso et al. 1987; Nyberg et al. 1998; Thomas and Machen 1991; Flemstrom and Garner 1982). This pathway was generally accepted as it not only allowed Cl^- to enter the cells, but at the same time provided a means to excrete HCO_3^-, which would in turn cause the cell interior to become acid and provide a continuing supply of protons for acid secretion. Recently, Lytle and colleagues identified an additional protein on the basolateral membrane the NKCC-1 using both immunofluorescence and functional studies (McDaniel et al. 2005; McDaniel and Lytle 1999) that could provide a source for Cl^- ions as well as K^+. In these studies the NKCC1 appeared to be active in the resting phase, and it was proposed to act as a salt loader at rest and could help the gland create a 'flushing' effect following acid secretion. Additional mouse data from this group support this idea (McDaniel et al. 2005)

Classical pathways for activation of gastric acid secretion

The classical or conventional pathways associated with acid secretion involve either hormonal or neuronal stimulation. Activation of either of these pathways results in activation of the H,KATPase and secretion of HCl from the gland.

Hormonal

In order for the parietal cell to secrete acid, a combination of 'activating hormones' must be released that will eventually lead to activation of the cell. The mode of action of these hormones (gastrin, histamine) has been the focus of a great deal of research (Sachs et al. 1997; Andersson et al. 1998; Waldum et al. 1998; Friis-Hansen 2002; Dockray et al. 2005). At present it is felt that in response to a food stimulus, G cells release gastrin that then has two effects: (a) the primary effect is to stimulate the enterochromaffin-like cell (ECL) to release histamine, which in turns binds to receptors on the basolateral membrane of the parietal cell and causes acid secretion, by translocation of the H^+,K^+ ATPase to the apical pole of the cell; (b) it has also been postulated that gastrin can also have some direct effects on acid secretion (Hersey and Sachs 1995; Sachs 2003; Forte and Nauss 1963; Forte et al. 1980), namely that a similar activation of acid secretion would occur at higher concentrations of serum gastrin.

Neuronal

In addition to hormonal stimulation of acid secretion, the stomach is also under the control of neuronal-induced acid secretion (Kasbekar et al. 1969; Zhou et al. 2003). In response to either olfactory stimulus or stretch-induced activation, neuronal receptors along the surface of the stomach release acetyl choline that directly acts on the parietal cells to increase cyclic AMP concentration and cause insertion and activation of the H^+,K^+ ATPase (Zhou et al. 2003).

Novel pathways for activation of gastric acid secretion

As discussed in the previous sections, the classical pathways for gastric acid secretion involved either hormonal (histamine, gastrin) or neuronal (acetyl choline) stimulation, which leads to a series of events that would induce acid secretion. Recently, an additional pathway has been identified that can induce acid secretion in a resting cell, or can lead to prolonged acid secretion in a stimulated parietal cell (Cheng et al. 1999; Geibel et al. 2001; Hebert et al. 2004; Dufner et al. 2005; Busque et al. 2005).

Calcium-sensing receptor

The calcium-sensing receptor CaSR belongs to a class of G protein coupled receptors that was originally identified in the parathyroid gland (Riccardi et al. 1996; Bai et al. 1996; Brown et al. 1993) whose activation could modulate calcium levels within these tissues.

Following the initial discovery, this receptor has been identified in a wide variety of species and tissues (Hebert et al. 2004; Brown and Hebert 1997; Hebert et al. 1997; Riccardi et al. 1996; Bai et al. 1996). We originally identified this protein in the parietal cells of the stomach (Cheng et al. 1999) and later showed that stimulation or inhibition of the receptor could directly modulate acid secretion either in the presence or absence of secretagogues (Geibel et al. 2001). During this same time period, Rutten and colleagues identified (Rutten et al. 1999) this same receptor in human G cells and demonstrated that activation of this protein leads to gastrin release. These two pieces of data show that the receptor is not only expressed in rats, mice and humans, but that it has at least two points of action: (a) parietal cells where its activation leads directly to acid secretion, and (b) G cells where activation of the receptor leads to gastrin release. Recently, we have evidence that an allosteric activation of the receptor occurs in the presence of certain amino acids (Busque et al. 2005) resulting in a prolonged and enhanced secretion of acid. These data are suggestive that as absorption of nutrients occurs, levels of blood amino acids will continue to rise thereby stimulating the receptor and prolonging the acid secretory phase after hormonal stimulation diminishes.

System L amino acid transporter

Recently we have identified another pathway that can modulate acid secretion, the system L amino acid transporter on the parietal cell. This transport protein allows the direct exchange of amino acids from the basolateral surface to the cytosol of the parietal cell with the end result being increased proton secretion in the absence of secretagogues.

The identification of these two additional pathways may further help to explain the difficulty in controlling the production of acid and the continuing increase in numbers of patients suffering from hypersecretory disease. These data strongly suggest that diets rich in protein and amino acid would lead to extended periods of the production of gastric acid, and could be partially responsible for the increase in gastro-esophageal reflux disease in the world population.

Other apical transport proteins

For many years the apical pole of the parietal cell was thought to only contain a Cl^- channel, K^+ channel, and the H^+, K^+-ATPase. As we have illustrated in other sections of this review, this has now been shown to no longer be the case. In addition to having identified multiple K^+ channels and potentially additional Cl^- channels, two other transport proteins have now been identified.

NHE-3

For many years there has been discussion of whether the parietal cell contained an additional apical protein that could act to flush the gland at the end of the secretory cycle of acid secretion. Recently we were able to provide evidence for this protein on the apical surface of parietal cells from rats (Kirchhoff et al. 2003). In this study we were able to show that the sodium–hydrogen exchanger type 3 (NHE-3) was active in resting cells and could move protons across the cell membrane. In these studies we showed that the protein was capable

of moving H^+ ions either out or into the cell in exchange for Na^+. As the electrochemical gradient following a cycle of acid secretion (lumen pH 1.0) would favour H^+ ions entering the cell in exchange for Na^+ ions, a removal of H^+ ions would occur from the lumen of the gland with an accumulation of Na^+ during the resting phase. The fact that intracellular pH is always slightly acidic in the parietal cell (pH_i 7.2–7.3) (Geibel et al. 2001; Kirchhoff et al. 2003), and that NaCl content in the lumen of the stomach, as well as pH, increases during the inactive phase would add credence to this theory.

PAT-1

Another interesting and controversial apical protein is the localization of a Cl/HCO3 exchanger PAT-1 (SLC26A6) at the apical surface of the parietal cell (Petrovic et al. 2002). The authors of this study presented evidence via immunolocalization that PAT-1 is located in close proximity to the gastric H^+,K^+-ATPase at the apical pole of the parietal cell. Furthermore they postulate that the protein may act to buffer the secretory vesicles when they are re-internalized following acid secretion.

Conclusion

Gastric acid secretion is and remains a complex process involving a variety of transport proteins and receptors. When the parietal cell goes from a resting mode to the secretory phase a dynamic remodelling of the epithelium (Forte et al. 1977; Duman and Forte 2003; Forte 2004; Duman et al. 2004) occurs which allows the cell to secrete a highly concentrated acid into the lumen of the gastric gland where the pH can fall to as low as ~1.0. It is interesting to note that in this caustic environment the parietal cell maintains the ability to recycle ions and to excrete ions without allowing for destruction of the cell. With recent advances in techniques, we are now able to determine that the apical recycling pathway for potassium may be composed of multiple K^+ channels allowing for the continuing entry and exit of K^+ in the parietal cell. In addition to these K^+ channels, there is now evidence that the apical surface also contains at least two other ion exchange proteins (NHE3, PAT1), although the exact role for either of these proteins is not completely understood.

The identification of the CaSR at the basolateral membrane has now highlighted an additional receptor pathway that can modulate acid secretion. Activation of this receptor by amino acids (Busque et al. 2005) provides evidence of an allosteric feedback loop in which receptor activation can maintain acid secretion and provide for a more complete ionization of calcium and other ions to aid in their absorption. This receptor may play an important role as an additional target to prevent the secretion of acid (see Fig. 3).

With the identification of these new channels, transporters, and receptors the possibility of developing new therapies against these additional targets may improve the chances of developing more effective long-term treatments of hypersecretory states of acid secretion, and potentially help to manage those individuals who are resistant to conventional therapies.

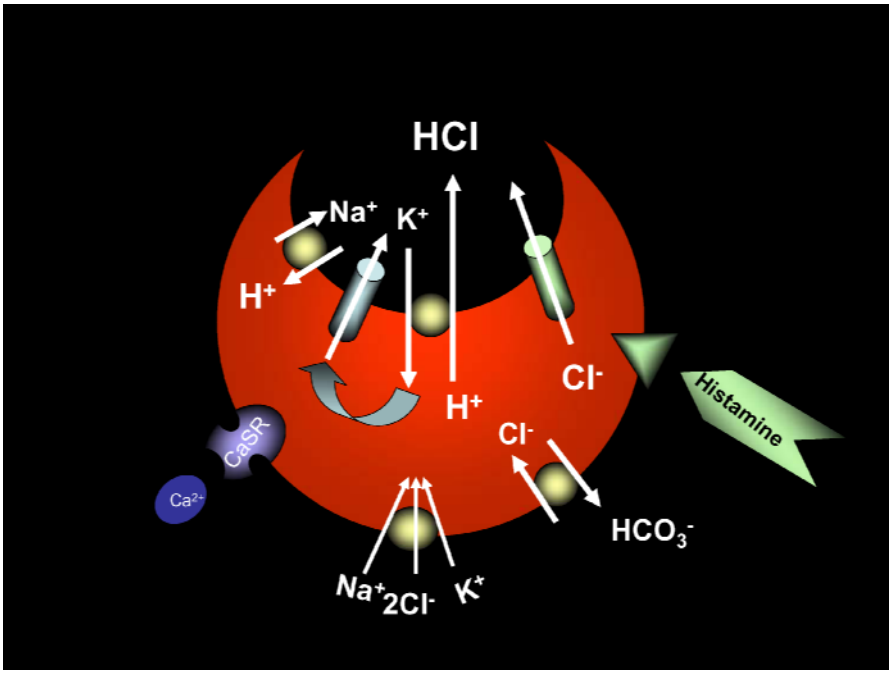

Fig. 3 A current model for the secretion of gastric acid by the parietal cell

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Structure–function relationship of the TRP channel superfamily

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Abstract Transient receptor potential (TRP) channels are involved in the perception of a wide range of physical and chemical stimuli, including temperature and osmolarity changes, light, pain, touch, taste and pheromones, and in the initiation of cellular responses thereupon. Since the last decade, rapid progress has been made in the identification and characterization of new members of the TRP superfamily. They constitute a large superfamily of cation channels that are expressed in almost all cell types in both invertebrates and vertebrates. This review summarizes and discusses the current knowledge on the TRP protein structure and its impact on the regulation of the channel function.

Introduction

Discovery of the first *Drosophila* transient receptor potential (TRP) channel involved in the response to light (Cosens and Manning 1969) led to the characterization of a large superfamily of cation channels that constitute important cation influx machinery in most vertebrate and invertebrate cell types. They are directly involved in thermo-, mechano-, chemo-, and nociception, responding to a wide variety of different physical and chemical stimuli (for recent reviews see Nilius and Voets 2005; Pedersen et al. 2005; Voets et al. 2005). Using structural homology as the criterion, the TRP channels have been classified into s127even subfamilies: TRPC (classical or canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), TRPA (ANKTM1 homologues) and TRPN (NOMP-C homologues) (Fig. 1) (Clapham 2003; Clapham et al. 2001, 2003; Vriens et al. 2004a).

TRP channels are intrinsic membrane proteins with six putative transmembrane spans (TM) and a cation-permeable pore region formed by a short hydrophobic stretch between TM5 and TM6 (Fig. 2). The length of the intracellular amino (N) and carboxy (C) termini and structural domains they encompass vary significantly between members of the TRP

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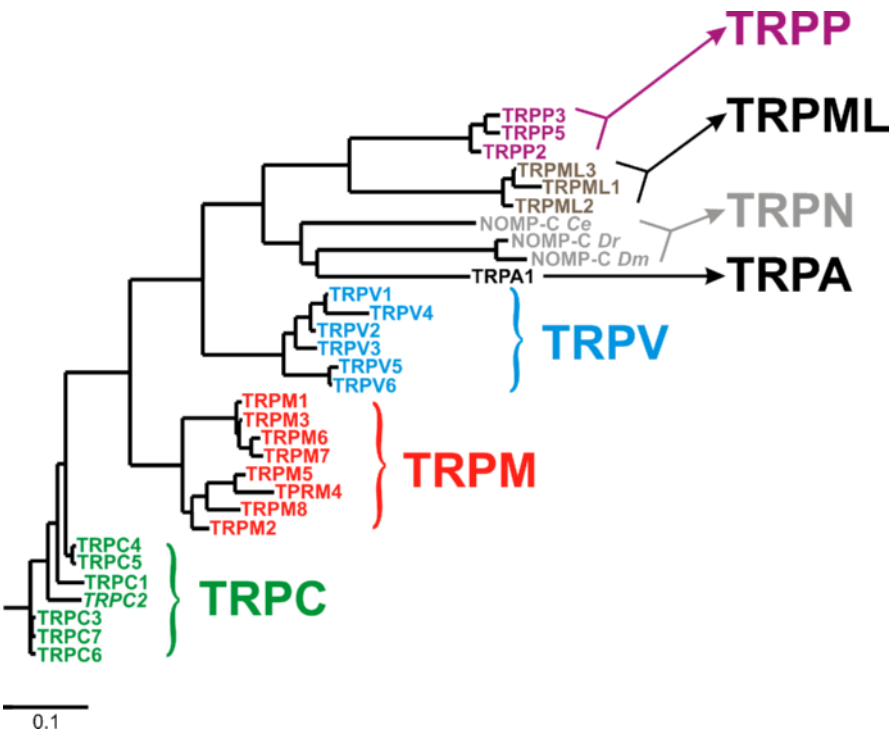

Fig. 1 Phylogenetic analysis of channels of the TRP superfamily; if not, annotated sequences of human channels were used for analysis. TRPC2 is a pseudogene in human and therefore the mouse channel sequence was used. The scale represents the evolutionary distance expressed in the number of substitutions per amino acid. *Dr*, *Danio rerio; Dm*, *Drosophila melanogaster; Ce, Caenorhabditis elegans*

channel subfamilies (Clapham 2003; Vriens et al. 2004a) (Table 1). These cytoplasmic parts play important roles in the regulation and modulation of channel function and trafficking. Functional TRP channels consist of four identical or similar TRP subunits (Garcia-Sanz et al. 2004; Hoenderop et al. 2003b; Kedei et al. 2001; Kuzhikandathil et al. 2001; Lintschinger et al. 2000; Strubing et al. 2001). In this review, we will discuss the current knowledge of the structure of TRP channels, with particular emphasis on structural elements involved in channel permeation and regulation. End Grabbed content

TRPs, a versatile superfamily of cation channels

The TRPC channel subfamily comprises the closest homologues of *Drosophila* TRP. There are seven TRPC channels in mammals. They are mainly phospholipase C (PLC) -dependent Ca^{2+} permeable cation channels formed by four either identical or different TRPC channel subunits (Clapham 2003; Clapham et al. 2003; Gudermann et al. 2004; Harteneck et al. 2000; Hofmann et al. 2002; Nilius 2003; Schilling and Goel 2004; Vazquez et al. 2004;). It is still controversial whether TRPCs are regulated by the depletion of intracellular Ca^{2+} stores (Grimaldi et al. 2003; Gudermann et al. 2004; Hofmann et al. 1999; Nilius 2003, 2004; Putney 2005; Strubing at al. 2001). Depending on the combination of TRPCs in the functional tetrameric channel, they play an important role in pheromone sensing (TRPC2;

Table 1 Putative motifs in the N- and C-terminal parts of human TRP channels

Channel	Potential motifs	Localization	Proposed function	References
TRPV1	Ankyrin (3 motifs)	201–230	*ND*	
Q8NER1[a]		248–277		
		333–362		
	N-terminal CaMBD	*189–222*[d]	Regulates capsaicin-activated currents	Rosenbaum et al. 2004
	PKC site (3 sites)	*502*	Regulates PMA enhancement of capsaicin-evoked currents	Bhave et al. 2003, Numazaki et al. 2002
		704	Regulates direct activation of TRPV1	Bhave et al. 2003
		800	Regulates PMA enhancement of capsaicin-evoked currents and direct activation of TRPV1	Bhave et al. 2003, Stowers et al. 2002
	PKA site	*116*	Interferes with the desensitization of capsaicin-evoked current	Bhave et al. 2002
	(3 sites)	*774*	Regulates channel desensitization	Mohapatra and Nau 2003
		820	Regulates channel desensitization	Mohapatra and Nau 2003
	TRP box	*684–721* or 697–702	Oligomerization	Garcia-Sanz et al. 2004
	C-terminal CaMBD	*768–802*	Regulates desensitization	Numazaki et al.2003
	PiP_2 binding site	*778–793*	Inhibition of the channel	Chuang et al. 2001, Prescott and Julius 2003
TRPV2	Ankyrin (3 motifs)	162–191	*ND*	
Q9Y5S1		208–237		
		293–323		
	TRP box	659–664	*ND*	
TRPV3	Ankyrin (3 motifs)	214–243	*ND*	
Q8NET8		261–291		
		340–369		
	TRP box	691–696	*ND*	
TRPV4	Ankyrin (3 motifs)	237–266	slight effect on hypotonic stimulation	Liedtke et al. 2000
Q9HBA0		284–313		
		369–398		
	PTK site	*253*[c]	Regulates channel function	Xu et al. 2003
			Phosphorylation of this residue has no effect	Vriens et al. 2004b
	TRP box	732–737	*ND*	
	CaMBD	*812–831*	Ca^{2+}-dependent potentiation	Strotmann et al. 2003
TRPV5	Ankyrin (5 motifs)	44–74	Regulates assembly, trafficking (residues 63–77)	Chuang et al. 2004
Q9NQA5		78–107		
		116–145		
		162–191		
		239–268		

Table 1 continued

Channel	Potential motifs	Localization	Proposed function	References
	TRP box	592–597	PiP_2 interaction site	Rohacs et al. 2005
	C terminus		Interaction with NHERF2, important for stabilization and targeting	Palmada et al. 2005
	C-terminal region	*596–601*	Regulates assembly, trafficking	Chuang et al. 2004
	C-terminal CaMBD	*650–729*	Regulates channel activity	Niemeyer et al. 2005
TRPV6	Ankyrin (5 motifs)	44–74	Tetramerization	Erler et al. 2004
Q9H1D0		78–107		
		116–145		
		162–191		
		239–268		
	N-terminal CaMBD	93–103	Regulates channel activity	Lambers et al. 2004
	TRP box	592–597	*ND*	
	Transmembrane CaMBD	*327–577*	Regulates channel activity	Lambers et al. 2004
	C-terminal CaMBD	691–711	Regulates channel activity	Niemeyer et al. 2005
				Lambers et al. 2005
TRPC1	Ankyrin (3 motifs)	46–75	Regulation of assembly, structure and channel function	Engelke et al. 2002
P48995		83–112		
		158–187		
	Coiled-coil domain	*212–267*[e]	Regulation of channel function	Engelke et al. 2002
	TRP box	659–664	*ND*	
	CaMBD1	*715–749*	Deletion has no effect	Singh et al. 2002
	CaMBD2	*758–793*	Affects SOCE, and Ca^{2+}-dependent inactivation	Singh et al. 2002
TRPC2[b]	Ankyrin (3 motifs)	46–75	*ND*	
Q9R244		83–112		
		158–187		
	TRP box	934–939	*ND*	
TRPC3	Ankyrin (4 motifs)	38–67	Regulates folding and trafficking	Wedel et al. 2003
Q13507		73–102		
		104–130		
		159–188		

Table 1 continued

Channel	Potential motifs	Localization	Proposed function	References
	PKG site (2 sites)	*11*	Effects TRPC3-mediated store-operated Ca^{2+} influx	Kwan et al. 2004
		263	Effects TRPC3-mediated store-operated Ca^{2+} influx	Kwan et al. 2004
	PKC site	*712*	Regulates channel activation	Trebak et al. 2005
	TRP box	684–689	*ND*	
	CIBR	777–797	Regulation of channel function	Wedel et al. 2003, Zhang et al. 2001
	C-terminal coiled-coil domain		Involved in trafficking	Wedel et al. 2003
TRPC4	Ankyrin (2 motifs)	69–98	*ND*	
Q9UBN4		141–170		
	TRP box	634–639	*ND*	
	CIBR	*688–759*	Interaction with NHERF and PLC-β1, important for allocation and regulation	Tang et al. 2000
		786–848		
	PDZ-binding motif	972–974	Regulates cell surface expression	Mery et al. 2002
TRPC5	Ankyrin (2 motifs)	69–98	*ND*	
Q9UL62		141–170		
	Coiled-coil domain	227–247	Interaction with stathmins	Greka et al. 2003
	TRP box	638–643	*ND*	
	PDZ-binding motif	*971–973*	Interaction with NHERF and PLC-β1, important for allocation and regulation	Tang et al. 2000
	PKC phosphorylation site	*972*	Phosphorylation regulates desensitization	Zhu et al. 2005
TRPC6	Ankyrin (4 motifs)	97–126	Interaction with MxA, regulates channel activity	Lussier et al. 2005
Q9Y210		132–161		
		163–189		
		218–247		
	TRP box	741–746	*ND*	
TRPC7	Ankyrin (4 motifs)	42–71	*ND*	
Q9HCX4		77–106		
		108–134		
		163–192		
	TRP box	686–691	*ND*	
TRPM1	TRP box	1019–1024	*ND*	
O75560				

Table 1 continued

Channel	Potential motifs	Localization	Proposed function	References
TRPM2	TRP box	1062–1067	*ND*	
O94759	Nudix hydrolase domain	1197–1503	ADPR pyrophosphatase	Perraud et al. 2001
TRPM3	TRP box	1051–1056	*ND*	
Q9HCF6				
TRPM4	TRP box	1057–1062	*ND*	
Q8TD43	C-terminal coiled-coil domain	*1136–1141*	Mediates the decavanadate effect	Nilius et al. 2004a
	PKC site (2 sites)	*1145* *1152*	Regulates Ca^{2+} sensitivity	Nilius et al. 2005b
	C-terminal CaMBD	*1076-1167*	Regulates Ca^{2+} sensitivity	Nilius et al. 2005b
TRPM5	TRP box	986–991	PiP_2 interaction site	Rohacs et al. 2005
Q9NY34				
TRPM6	TRP box	1083–1088	*ND*	
Q9BX84	PLIK	1708–1986	*ND*	
TRPM7	TRP box	1110–1115	*ND*	
Q96QT4	PLIK[d]	1554–1829	Regulation of channel activity	Runnels et al. 2001 Runnels et al. 2002
			Assembly and subcellular localization	Matsushita et al. 2005, Nadler et al. 2001 Runnels et al. 2001
TRPM8	TRP box	993–998	PiP_2 interaction site	Rohacs et al. 2005
Q7Z2W7				
TRPP2	Polycystin motif	316–328	*ND*	
Q13563	EF hand	750–785	*ND*	
	Coiled-coil domain	between *742-871*	Homodimerization and interaction with TRPP1	Tsiokas et al. 1997
	ER localization signal	*787–820*	ER retention	Cai et al. 1999
TRPP3	Polycystin motif	195–207	*ND*	
Q9POL9	EF hand	*637–665*	*ND*	Nomura et al.1998
	Coiled-coil domain	*656–687*	*ND*	
TRPP5	Polycystin motif	126–138	*ND*	
Q9NZM6				
TRPML1	Proline rich (2 domains)	*28–36*	*ND*	Sun et al. 2000
Q9GZU1		*197–205*		
	Nuclear localization motif	*43–60*	*ND*	
	Lipase serine active site	*104–114*	*ND*	
	C-terminal di-leucine motif	*563–566* *577–580*	Late endosomal/lysosomal targeting	

Table 1 continued

Channel	Potential motifs	Localization	Proposed function	References
TRPML2 **Q81ZK6** and TRPML3 **Q8TDD5**			Both channels remain to be fully characterized	
TRPA1 **O75762**	Ankyrin (15 motifs)	62–92 97–126 130–160 164–193 197–226 138–267 271–301 308–337 341–370 412–441 445–474 481–510 513–542 547–576 579–609	Formation of the gating spring	Corey et al. 2004

Numbers in italics correspond to the published data, while others are determined by Swiss-PROT. *ND*, not determined
[a] Accession number
[b] TRPC2 is pseudogene in human, therefore mouse TRPC2 sequence was used
[c] Function is still controversial
[d] Rat
[e] Mouse

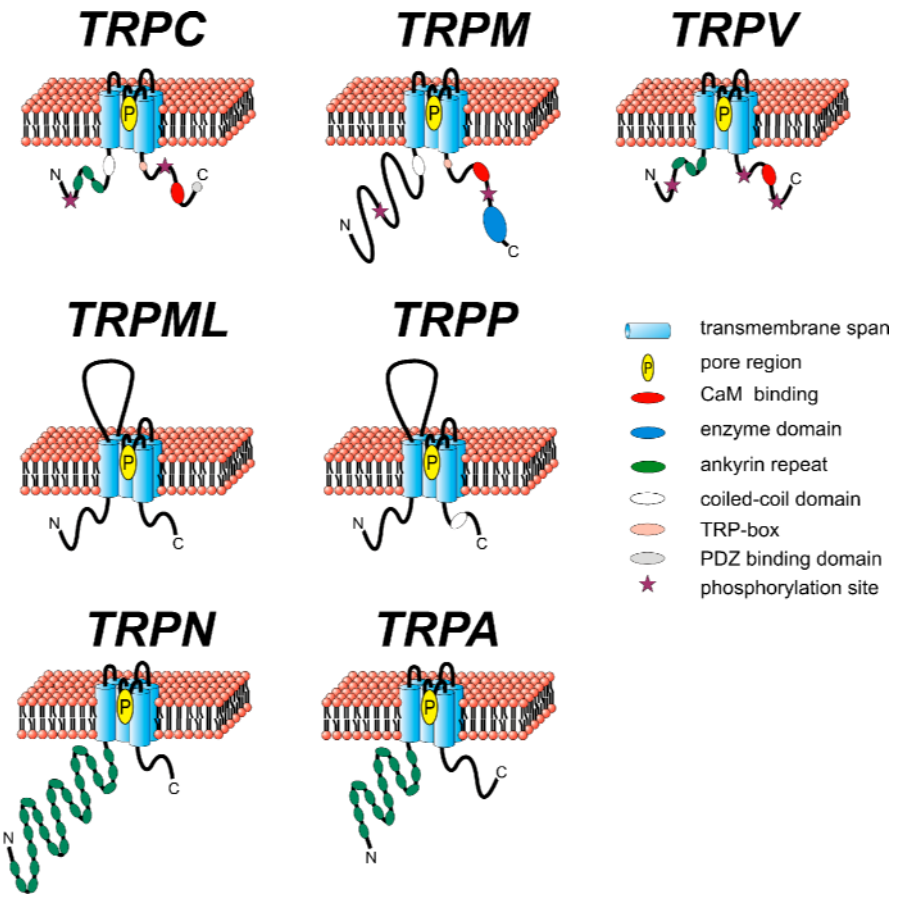

Fig. 2 Schematic representation of the structural topology of channels from the TRP-related subfamilies. The transmembrane segments are similar in all TRP channels. The putative pore region is localized between TM5 and TM6 and its length and amino acid composition are variable in different subfamily members. Only the most representative domains are annotated and lengths of the N- and C-termini are approximated

note that the human TRPC2 is a pseudogene), vasoregulation (TRPC3/4/5), signaling in the central nervous system (TRPC1/3/4), and functioning of smooth muscle cells (TRPC3/6/7) (Freichel et al. 2001; Kim et al. 2003; Lucas et al. 2003; Sakura and Ashcroft 1997; Stowers et al. 2002; Strubing et al. 2001; Tiruppathi et al. 2002). TRPC1 may also function as a stretch-activated channel involved in cellular mechanosensitivity (Maroto et al. 2005).

In the subfamily of TRPV channels, six mammalian homologues have been classified. TRPV1/2/3/4 are permeable to Ca^{2+} with a rather low selectivity for divalent and monovalent cations (Benham et al. 2002; Gunthorpe et al. 2002; Voets and Nilius 2003; Voets et al. 2002). The vanilloid receptor, TRPV1, mediates nociception and is involved in the detection and integration of thermal and diverse chemical stimuli (e.g., vanilloids, endovanilloids, and anandamide) (Caterina et al. 2000; Jordt and Julius 2002). TRPV2 and TRPV3 are activated in the noxious and warm heat range, respectively (Kanzaki et al. 1999; Peier et al. 2002b; Smith et al. 2002; Xu et al. 2002). TRPV4 contributes to nociception and osmo- and warmth sensation, and is activated by ligands such as α-phorbols or endogenous agonists such as epoxyeicosatrienoic acids (Liedtke et al. 2000; Liedtke and Friedman 2003; Nilius

et al. 2004b; Vriens et al. 2004b; Watanabe et al. 2002a, 2003). TRPV5 and TRPV6, the only highly Ca^{2+}-selective TRP channels, play an important role in Ca^{2+} reabsorption in kidney and intestine (den Dekker et al. 2003; Hoenderop et al. 2002a, 2002b, 2003a, 2003b; Nijenhuis et al. 2003; Vennekens et al. 2000, 2001a, 2001b).

A third subfamily of TRP-related channels includes close homologues of melastatin. Melastatin was originally identified based on its higher expression in nonmetastatic compared to highly metastatic melanoma cells (Duncan et al. 1998). The TRPM subfamily in mammals comprises eight members that are involved in processes as different as Mg^{2+} homeostasis (TRPM6, TRPM7 [Nadler et al. 2001; Schlingmann et al. 2002; Voets et al. 2004c; Walder et al. 2002]), taste detection (TRPM5 [Perez et al. 2002; Zhang et al. 2003]), cell proliferation (TRPM7 [Nadler et al. 2001]), and noxious cold sensing (TRPM8 [McKemy et al. 2002; Peier et al. 2002a; Voets et al. 2004a]). Except for TRPM1, the permeation properties of TRPMs are relatively well described. The Ca^{2+}-activated TRPM4/5 channels are the only Ca^{2+}-impermeable TRPs identified so far (Hofmann et al. 2003; Launay et al. 2002; Nilius et al. 2003a; Prawitt et al. 2003). TRPM2/3 and TRPM8 are Ca^{2+}-permeable with rather low Ca^{2+} selectivity (Grimm et al. 2003; Hara et al. 2002; Lee et al. 2003; McKemy et al. 2002; Peier et al. 2002a; Perraud et al. 2001; Sano et al. 2001), whereas TRPM6/7 are relatively highly permeable for divalent cations, especially for Mg^{2+} (Monteilh-Zoller et al. 2003; Nadler et al. 2001; Voets et al. 2003; Voets and Nilius 2003).

The polycystin subfamily, TRPP, is named after its founding member, PKD2, which was discovered as one of the genetic determinants of autosomal dominant polycystic kidney disease (ADPKD) (Mochizuki et al. 1996). There are three mammalian TRPP channels: TRPP2 (PKD2), TRPP3 (PKD2L1), and TRPP5 (PKD2L2) (Cai et al. 1999; Guo et al. 2000; Nomura et al. 1998; Veldhuisen et al. 1999; Wu et al. 1998). Functional expression of human TRPP2 channel in the plasma membrane depends on the interaction with PKD1, a large plasma membrane protein with 11 putative TMs (Hanaoka et al. 2000; Qian et al. 1997; Tsiokas et al. 1997). This interaction may occur via a putative coiled-coil domain in the C terminus of TRPP2. In the absence of PKD1, TRPP2 may function as an intracellular Ca^{2+} release channel (Koulen et al. 2002). There is evidence that TRPP channels may function as mechano-sensors in ciliated epithelial cells and might be important for organogenesis (Nauli et al. 2003; Stayner and Zhou 2001).

Mutations to TRPML1 (mucolipin-1; MCOLN1) lead to mucolipidosis type IV (MLIV), an autosomal recessive, neurodegenerative, lysosomal storage disorder characterized by psychomotor retardation and ophthalmological abnormalities, including corneal opacities, retinal degeneration, and strabismus (Bargal et al. 2000; Bassi et al. 2000; Berman et al. 1974). Human TRPML1 expressed in *Xenopus laevis* oocytes functions as a Ca^{2+}-permeable cation channel that is modulated by changes in Ca^{2+} concentrations (LaPlante et al. 2002). Mutations of mouse TRPML3 (MCOLN3) lead to deafness and defects of hair cell and pigmentation (the varitint-waddler mouse) (Di Palma et al. 2002). There are some indications that mutations in genes encoding TRPML2 (MCOLN2) and TRPML3 may be involved in hereditary and/or sporadic neurosensory disorders in humans (Di Palma et al. 2002; and for a general review see Nilius et al. 2005d).

TRPA and TRPN subfamilies are very closely related and poorly represented in vertebrates. ANKTM1 or TRPA1 is the only TRPA subfamily member characterized in vertebrates so far (Corey 2003; Story et al. 2003). Mammalian TRPA1, a Ca^{2+}-permeable, nonselective cation channel, is activated by noxious cold, bradykinin, cannabinoids, and several pungent compounds such as the isothiocyanates that are present in wasabi and mustard, cinnamaldehyde, and allicin (Bandell et al. 2004; Jordt et al. 2004; Macpherson et al. 2005; Story et al. 2003). It is highly expressed in hair bundles of sensory hair cells where it may

function as a mechanosensory transduction channel involved in the hearing process (Corey et al. 2004; Lin and Corey 2005).

The TRPN subfamily is named after the *n*o *m*echanoreceptor *p*otential *C* (NOMP-C) channel from *Drosophila*, which plays a crucial role in mechanosensation in processes such as hearing, balance, proprioception, and touch (Kernan et al. 1994; Walker et al. 2000). No obvious homologues of NOMP-C are present in the human genome. So far, the only vertebrate TRPN family member that has been identified is NOMP-C from zebrafish (Sidi et al. 2003). Mutations in NOMP-C of zebrafish larvae lead to impaired hair cell mechanotransduction and the loss of microphonic potentials (Sidi et al. 2003).

Transmembrane segments: a functional backbone of TRP channels

The transmembrane segments are the most conserved structures in all TRP channels. Although the number of hydrophobic regions can vary from one TRP channel to another, it is generally believed that only six α-helices are able to span membranes. By analogy to K^+ channels, TM5 and TM6 seem to play a central role and directly contribute to formation of the channel pore (see "Functional insights into the pore region of TRP channels"). In contrast to classical voltage-gated cation channels, TM4 contains only a few positively charged residues, which complicates determination of the residues responsible for voltage-dependent activation of TRP channels.

Relatively few reports describe the functional impact of TM1–4 segments in regulation of the TRP channel function. The first insight in the functional role of TM2–4 came from a study on TRPV1 aiming at molecular determination of the specific sites that bind vanilloid compounds such as capsaicin or the endogenous TRPV1 agonist, anandamide (Gavva et al. 2004; Jordt and Julius 2002). Jordt and Julius (2002) showed that substitutions of a conserved tyrosine residue (Y^{511}), which is located within or adjacent to TM3, by phenylalanine, alanine or cysteine led to either a selective loss of capsaicin sensitivity or reduced capsaicin-activated currents. Similar effects were also induced by mutations to a neighboring seine (S^{512}) or R^{491} in the TM2. Thus, a proposed model of vanilloid binding pocket comprises an aromatic residue, Y^{511}, which interacts with the vanillyl-moiety of capsaicin on the cytosolic face of the membrane. The other residues, such as polar S^{512} or R^{491}, may interact with capsaicin via hydrogen bonds, whereas lipophilic residues in TM3 can be involved in stabilization via hydrophobic interactions with the aliphatic moiety of capsaicin within the plane of the membrane (Jordt and Julius 2002). This model was partially confirmed by Gavva et al. (2004), who investigated mechanisms of capsaicin insensitivity of rabbit TRPV1. Using either human/rabbit or rat/rabbit chimeras, they demonstrated that apart from Y^{511} additional residues in TM4, M^{547}, and T^{550} directly contribute to vanilloid binding. In contrast to Jordt and Julius, they propose that T^{550}, W^{549}, and M^{547} may be involved in interaction with the vanilloid moiety, whereas the aliphatic tail of capsaicin binds to Y^{511} (Gavva et al. 2004). This alternative model suggests that observed differences in affinity of ligands with longer (higher affinity) and shorter (lower affinity) aliphatic tails may be explained by their abilities to interact with Y^{511}. Both models are still preliminary and require additional biochemical and structural data for validation. Moreover, they do not explain why mutations of N- and C-terminal residues, R^{114} and D^{761}, in TRPV1 result in loss of capsaicin sensitivity (Jung et al. 2002; Vlachova et al. 2003).

Using sequence homology to TRPV1, a tyrosine important for ligand activation (Y^{555}) in TM3 has also been identified in TRPV4, a channel that is activated by a broad range

of stimuli such as osmotic cell swelling, heat, phorbol esters, and arachidonic acid (AA) (Vriens et al. 2004b; Watanabe et al. 2002a, 2002b, 2003). Mutation of Y^{555} to nonaromatic residues resulted in a strong decrease of TRPV4 activation by 4α-phorbol 12, 13-dideconoate (4αPDD) and heat, but does not affect activation by cell swelling or AA (Vriens et al. 2004b). This suggests that activation of TRPV4 by phorbol esters and heat both occur via a pathway that critically depends on an aromatic residue in TM3. Very likely other residues in TM2 or TM4, which remain to be identified, may also be involved in ligand binding in TRPV4.

Functional insights into the pore region of TRP channels

Ion channels are pore-forming transmembrane proteins that allow permeation of ions through biological membranes. The structure of the channel pore is crucial for determination of the ion permeation and selectivity properties of particular channels. In contrast to other families of ion channels, data concerning structure and localization of TRP channel pores are rather limited and only concerns TRPV, TRPC, and TRPM subfamilies (Fig. 3). Nevertheless, all available functional and theoretical data strongly support the general notion that the linker region between TM5 and TM6 is the pore-forming part in all channels of the TRP superfamily (Owsianik et al. 2006).

TRPVs

The structure–function analysis of TRPV channel pores is the most advanced among all TRP subfamilies. In all mammalian TRPVs, TM5–6 linker regions show significant sequence homology with the selectivity filter of the prokaryotic potassium channel KcsA, whose crystal structure has been determined at 2-Å resolution (Doyle et al. 1998; Zhou et al. 2001a). Mutations to negatively charged residues, D^{546} of TRPV1 and corresponding D^{682} of TRPV4, strongly reduce the permeability for Ca^{2+} and Mg^{2+} and decrease the affinity of the channels to the voltage-dependent pore blocker Ruthenium Red (Garcia-Martinez et al. 2000; Voets et al. 2002). Additionally in TRPV4, mutation of neighboring residue D^{672} further reduces the selectivity for divalent and also changes the relative permeability for monovalent cations, whereas the substitution of M^{680} with a negatively charged amino acid abolishes Ca^{2+} and Mg^{2+} permeability (for predicted localization of these residues see the scheme in Fig. 3B). Mutation of the only basic pore residue in TRPV4, Lys^{675}, did not significantly change the permeation properties of the channel (Voets et al. 2002). These results indicate that the putative TRPV1/2/3/4 pore motif, GM(L)GD, determines permeation properties of the channels and is functionally homologous to the GYGD signature sequence in the selectivity filter of K^+ channels.

Permeation properties of TRPV5/6 are also determined by the aspartate residues in the putative selectivity filter (Hoenderop et al. 2003b; Nilius et al. 2001; Voets et al. 2001, 2003). Aspartate-to-alanine mutations at position D^{542} of TRPV5 and D^{541} of TRPV6 result in the loss of Ca^{2+} permeation, Ca^{2+}-dependent current decay, and block by extracellular Mg^{2+} or Cd^{2+}, whereas permeation of monovalent cations remains unchanged. Other negatively charged residues in the pore region of TRPV5, E^{535} and D^{550}, have less impact on pore properties, whereas E^{522}, located N-terminal of the pore helix, functions as a putative pH sensor, regulating pH-dependent permeation properties of TRPV5/6 (Vennekens et al. 2001a; Yeh et

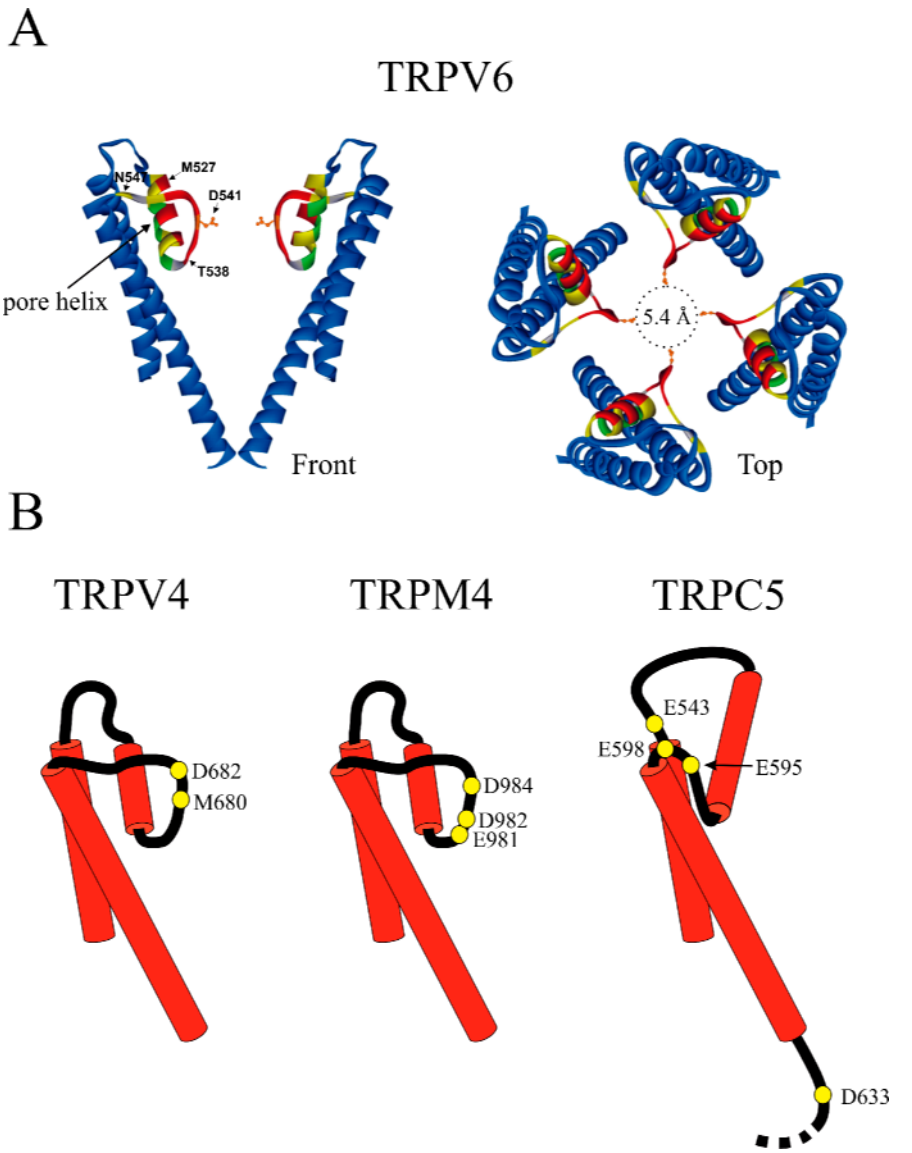

Fig. 3A, B Predicted topologies of the pore region of TRPV, TRPM and TRPC channels. **A** Structural model of the TRPV6 pore region, looking sideways at two opposite subunits (*left*) or looking down from the external solution to the complete homotetrameric channel. At the narrowest point, the pore is formed by the acidic side chain of Asp^{541} (*orange*) and has a diameter of 5.4 Å. *Blue* residues correspond to the residues in TM5 and TM6 and amino acids that were subjected to SCAM analysis (residues P^{526} to N^{547}) are colored in *green*, *yellow*, *red*, or *gray*. Residues in *red* reacted rapidly to Ag^+ (reaction rate $>5.10^6$ $M^{-1}s^{-1}$), residues in *yellow* reacted with Ag^+ at a rate $<5.10^6$ $M^{-1}s^{-1}$, and residues in *green* did not show significant reactivity to Ag^+. Residues where cysteine substitution resulted in nonfunctional channels are colored in *gray*. (Adapted from Voets et al. 2004b, with copyright permission from *The American Society for Biochemistry and Molecular Biology*). **B** Schematic representation of crucial residues in putative selectivity filters of TRPV4, TRPM4, and TRPC5 channels (see text for details)

al. 2003). Using the substituted cysteine accessibility method (SCAM), a more detailed pore structure of TRPV5/6 has been obtained (Dodier et al. 2004; Voets et al. 2004b). Cysteines introduced in residues that are N-terminal to $D^{542/541}$ show a cyclic pattern of reactivity, indicating that these residues form a pore helix similar to that in the KcsA crystal structure (Doyle et al. 1998; Zhou et al. 2001a). In TRPV6, the pore helix is followed by the selectivity filter with a diameter of approximately 5.4 Å at its narrowest point, as assessed by measurements of permeability to cations of increasing size (Voets et al. 2004b). The apparent pore diameter of TRPV6 increases significantly when D^{541} is substituted by amino acids with a shorter side chain, demonstrating that this aspartate residue shapes the narrowest part of the selectivity filter and contributes to the sieving properties of the pore (Voets et al. 2004b) (Fig. 3A). Thus, these results strongly indicate that selectivity and permeation properties of TRPV5/6 depend on a ring of four aspartate residues in the channel pore, similar to the ring of four aspartates and/or glutamate residues in the pore of voltage-gated Ca^{2+} channels (Heinemann et al. 1992; Talavera et al. 2001; Yang et al. 1993).

TRPCs

Unlike TRPV channels, the TM5–TM6 region of TRPCs does not share significant sequence homology with the pore region of K^+ channels. The most direct evidence for the location of the pore region of TRPC channels comes from functional identification of TRPC1 as a store-operated Ca^{2+} channel (SOCC) (Liu et al. 2003) and studies of La^{3+} potentiation of TRPC5 (Jung et al. 2003).

TRPC1 can potentially form eight hydrophobic α-helices but only six of them are believed to span the membrane (Dohke et al. 2004). One of the non-membrane-spanning α-helices is located in the region between TM5 and TM6 (note that the TMs are numbered differently than proposed in Dohke et al. 2004) and seems to form a pore helix similar to that in KcsA and TRPV5/6. Mutations to all seven negatively charged residues in the TM5–TM6 region of TRPC1 (D to N and E to Q) result in decreased Ca^{2+} but intact Na^+ currents through TRPC1, and induce shifts in the reversal potential (Liu et al. 2003). Interestingly, the crucial residues, E^{576}, D^{581}, and E^{615}, are located in the distal parts of the putative pore mouth, suggesting a different pore structure than that of the TRPV subfamily.

In analogy to TRPC1, neutralization of 3 of the 5 glutamates in the loop between TM5 and TM6 of TRPC5, E^{543}, E^{595}, and E^{598}, lead to a loss of La^{3+} potentiation (Jung et al. 2003). Moreover, the E^{595}/E^{598} double mutant shows altered single channel properties. Surprisingly, mutations of either E^{559} or E^{570} located in the central part of this loop do not affect the channel properties. More recently, Obukhov and Nowycky (2005) demonstrated that D^{633}, which is situated intracellularly between the end of TM6 and the TRP box, is a crucial residue for current block by intracellular Mg^{2+} in TRPC5 homotetrameric channels. Mutations of D^{633} to either noncharged or positively charged residues display markedly reduced inward currents and decreased voltage-dependent Mg^{2+} block (Obukhov and Nowycky 2005). In summary, all these results indicate that negatively charged residues that appear to be located close to but exterior of the pore region control permeation properties of TRPC1/5 channels (for predicted location of these residues see the scheme in Fig. 3B).

TRPMs

In all members of the TRPM subfamily, the TM5–TM6 region is highly conserved and shares limited homology to pore regions of KcsA and TRPVs channels. It consists of a putative hydrophobic pore helix, followed by an invariant aspartate, which seems to be located in the selectivity filter (Perraud et al. 2003). Other conserved negatively charged residues between the putative pore helix and the fully conserved aspartate may form a cluster of negative charges that contributes to the pore properties of TRPM channels. Recently, taking advantage of the theoretical prediction of the putative pore region of TRPM channels, Nilius et al. identified several residues responsible for the permeation properties of the TRPM4 pore and its blockade by intracellular spermine (Nilius et al. 2005a). Substitution of E^{981}DMDVA^{986} residues of TRPM4 with the selectivity filter of TRPV6 (T^{538}IIDGP^{543}) results in a functional channel that combines the gating hallmarks of TRPM4, such as activation by Ca^{2+} and voltage dependence, with TRPV6-like sensitivity to channel block by extracellular Ca^{2+} and Mg^{2+}. Furthermore, neutralization of E^{981} by alanine abolishes TRPM4 affinity to block by spermine, strongly indicating that E^{981} is placed in the inner part of the pore where it is exposed to intracellular spermine (Nilius et al. 2005a). Mutations of the neighboring aspartates, D^{982} and D^{984}, strongly affect the rundown and voltage dependence of the channel, whereas substitution of Gln^{977} by a glutamate, the site occupied by a negatively charged residue in divalent cation-permeable TRPMs, modifies monovalent cation permeability and leads to the channel with moderate Ca^{2+} permeability (for predicted location of these residues, see the scheme in Fig. 3B). These experiments provide, so far, the strongest direct proof that the TRPM4 selectivity filter is located between TM5 and TM6.

Additional information on the pore region of TRPM channels comes from functional analysis of TRPM3 splice variants. TRPM3α1–5 differ in the length of the putative pore region as one splice site is located in the TM5–TM6 loop (Oberwinkler et al. 2005). TRPM3α1, a variant that encompasses an optional stretch of 12 amino acids following the invariant aspartate, forms a channel with low permeability for divalent cations, whereas TRPM3α2, which lacks this stretch of amino acids, has more than tenfold higher permeability to Ca^{2+} and Mg^{2+} and is sensitive to block by extracellular monovalent cations. Again, these data indicate that TM5–TM6 region covers the pore region of the TRPM channels.

Intracellular determinants implicated in function of TRP channels

TRP domain

Discovery of mammalian members of the TRPC subfamily revealed the existence of a highly conserved structure localized in the C terminus close to TM6 (Bhave et al. 2003; Clapham et al. 2001; Minke and Cook 2002; Montell et al. 2002a; Prescott and Julius 2003). This so-called TRP domain consists of 25 amino acids, six of which are referred to as a TRP box. This TRP box has been postulated to serve as a putative signature of the TRP channel superfamily, but in view of the latest TRP channel classification (Clapham et al. 2003; Montell et al. 2002b), the use of the TRP box as a hallmark of the TRP superfamily has to be revised, as it is not conserved in TRPP, TRPML, TRPA, and TRPN subfamilies.

In TRPC channels, the TRP box is fully conserved and characterized by the specific amino acid sequence, EWKFAR. In TRPVs and TRPMs, the conservation of the TRP box is very low, going from IWxLQx (with x = K, R, or W) for TRPV1–4 and LWRAQx (with x =

V or I) for TRPV5–6, to xWKFQR (with x = I, V, or F) for TRPM1–3/5/7–8, YWKAQR for TRPM4, and LWKYNR for TRPM6. Surprisingly, nothing is known about functional role of the TRP box in TRPC channels. It has been recently shown that in TRPV1 the putative TRP-box motif (amino acids from D^{684} to R^{721}) may function as an association domain involved in oligomerization of the channel (Garcia-Sanz et al. 2004). Biochemical and immunological analysis indicate that self-association of recombinant C-termini of TRPV1 as well as of full-length TRPV1 monomers is blocked when the segment between D^{684} and R^{721} is deleted. Additionally, such deletion in a poreless TRPV1 mutant subunit suppressed its robust dominant-negative phenotype. These data suggest that the TRP-box region may act as a molecular determinant responsible for tetramerization of TRPV1 subunits into functional channels (Garcia-Sanz et al. 2004).

Another interesting insight in the function of the TRP box comes from the study of phosphatidylinositol 4,5-bisphosphate (PIP_2) -dependent activation of TRP channels (Rohacs et al. 2005). Mutations at conserved positively charged amino acid residues in the TRP-box, K^{995}, R^{998}, and R^{1008} of TRPM8 decrease the sensitivity of the channel to PIP_2 and enhanced channel inhibition by PIP_2 depletion. Similar results were obtained when analogue mutations were introduced into the TRP domain of TRPM5 (R^{1006}) and TRPV5 (R^{599}) (Rohacs et al. 2005). All these observations suggest that the positively charged residues in the TRP box are important determinants for interaction with PIP_2 and that regulation by PIP_2 is a common feature of members of the TRP channel family.

Ankyrin repeats

Most of the TRP channels contain N-terminal ankyrin (ANK) repeats, which are 33-residue motifs consisting of pairs of antiparallel α-helices connected by β-hairpin motifs (for review, see Sedgwick and Smerdon 1999). ANK repeats are involved in specific protein–protein interactions and can interconnect membrane proteins with the spectrin-actin-based membrane skeleton (Denker and Barber 2002). The number of ANK repeats in the N terminus of TRP channels varies between different members of the same subfamily. TRPCs and TRPVs typically possess three or four ANK motifs, compared to 14–15 in TRPAs and approximately 29 (!) in TRPNs.

The role of ANK repeats in TRP channels is still unclear and controversial. In TRPC1, deletion of the region containing all three ANK repeats had no effect on dimerization of the channel, as shown by the yeast two-hybrid analysis (Engelke et al. 2002). Although such mutant channels are inserted correctly in the membrane, they do not form functional TRPC1 channels, suggesting that ANK repeats may interact with interaction partners that are needed for the correct assembly of the quaternary channel structure or regulation of channel function (Engelke et al. 2002). In contrast, a similar N-terminal deletion of the region comprising all three ANK repeats of TRPC3 results in retention of the truncated channel in intracellular compartments. Truncation of the N terminus up to the first ANK repeat does not influence channel function or targeting to the plasma membrane, indicating that these motifs may be involved in channel trafficking (Wedel et al. 2003). In TRPV6, which contains six ANK repeats (Peng et al. 2000), only the third ANK motif is a key determinant of tetramerization (Erler et al. 2004). It may serve as an initiator of the molecular zippering process that proceeds after the fifth ANK repeat, creating an intracellular anchor that is necessary for a functional assembly of TRPV6 subunits (Erler et al. 2004). Surprisingly, complete deletion of the TRPV4 N terminus including three ANK repeats had no effect on targeting the channel to the plasma membrane (Liedtke et al. 2000). Electrophysiological measurements

also show no significant differences in the responses of the TRPV4 N-terminal truncants to hypotonic stimulation, except that cells expressing the truncated protein respond less robustly than intact TRPV4 in the first 60 s after hypotonic stimulation (Liedtke et al. 2000).

More recently, an interesting hypothesis for a functional role of ANK repeats in TRPA and TRPN channels has been proposed. In mechanosensitive TRPA1 and TRPN1 channels, mechanical stress may be transduced to these channels via their cytosolic tails, which may be connected to cytoskeletal elements (Corey et al. 2004; Howard and Bechstedt 2004; Lin and Corey 2005; Sotomayor et al. 2005). Crystallographic studies have shown that multiple ankyrin repeats can form a helical structure, which may act as a gating spring. Theoretical calculations of the stiffness of such an ankyrin helix yield values of around 1–5 mN/m, which is on the same order of magnitude as the experimentally deduced stiffness of gating springs in vertebrate hair cells (Howard and Bechstedt 2004; Sotomayor et al. 2005).

Coiled-coil domain

Coiled-coil domains are protein oligomerization motifs that consist of two or more alpha helices that twist around one another to form a supercoil (Burkhard et al. 2001). Peptides with the capacity to form coiled coils are characterized by a heptad repeat pattern in which residues in the first and fourth position are hydrophobic, and residues in the fifth and seventh position are predominantly charged or polar. Analysis of TRP channel sequences reveals the presence of putative coiled-coil domains in TRPCs and TRPMs, but the function of these predicted motifs has not yet been extensively studied. Using yeast two-hybrid experiments, it has been shown that the N-terminal coiled-coil structure of TRPC1 facilitates a homomerization process (Engelke et al. 2002). Although the mutant lacking the coiled-coil region can be correctly inserted in the membrane, Ca^{2+} influx in cells expressing this mutant is significantly reduced compared to wild-type TRPC1. These data suggest that the N-terminal coiled-coil region is involved in regulation of the TRPC1 channel function via interaction with other proteins (Engelke et al. 2002).

More recently, Nilius et al. showed that deletion of the R^{1136}ARDKR^{1141} region in the putative C-terminal coiled-coil domain of TRPM4 eliminates the effect of decavanadate on TRPM4 activation (Nilius et al. 2004a). Interestingly, this site also shows some similarities with the pleckstrin domain of PLC (K-X_{3-11}-R/K-X-R-Hyd-Hyd; where Hyd corresponds to any hydrophobic amino acid), which mediates interaction with second messenger lipids such as PIP_2 (Harlan et al. 1994, 1995).

PDZ-binding domain

PDZ domains (named after the three proteins in which this motif was first described: the postsynaptic density protein PSD, disc-large tumor suppressor, and the tight junction protein ZO-1) are protein interaction domains that are often found in multidomain scaffolding proteins. PDZ-containing scaffolds assemble specific proteins into large molecular complexes at defined locations in the cell. They are specialized in binding to short peptide motifs, PDZ-binding motifs, at the extreme C-termini of other proteins (Kim and Sheng 2004). In the TRP channel superfamily, putative PDZ-binding domains have only been found in TRPC and TRPV subfamilies. In the *Drosophila* TRPCs, TRP and TRP-like (TRPL), the C terminus interacts with the PDZ domain-containing protein INAD (Tsunoda and Zuker 1999), which enables heteromultimerization of these channels in the signaling complex (Chevesich et al.

1997; Huber et al. 1998; Leung et al. 2000; Li and Montell 2000; Xu et al. 1998). The PDZ-binding motif of TRPC4 and TRPC5 is formed by a C-terminal stretch of five amino acids, VTTRL. The presence of the TRL sequence in this motif is essential for the interaction of TRPC4/5 with PDZ domain-containing proteins such as hydrogen exchanger regulating factor (NHERF) or ezrin/moesin/radixin-binding phosphoprotein 50 (EBP50) (Lee-Kwon et al. 2005; Mery et al. 2002; Tang et al. 2000). As shown by co-precipitation experiments, TRPC4 and TRPC5 are able to co-assemble with NHERF as well as with NHERF-interacting partner, PLC-β1, suggesting that this interaction forms an important mechanism for allocation and regulation of the channels (Tang et al. 2000). Furthermore, it has been also shown that deletion of the PDZ-binding motif in TRPC4 strongly reduces expression of the channel at the cell surface and also changes its general distribution in the cell membranes to a predominant expression in cell outgrowths (Mery et al. 2002).

No evident PDZ-binding domains have been identified in the C terminus of TRPVs, but coexpression studies in *Xenopus* oocytes revealed that TRPV5 conductance is activated by the scaffold protein NHERF2 by increasing the channel abundance at the plasma membrane. This stimulatory effect requires the presence of the serum and glucocorticoid inducible kinase SGK1 (Embark et al. 2004). The interaction of NHERF2 and TRPV5 is a Ca^{2+}-independent process that requires the second PDZ domain of NHERF2 and the C-tail of TRPV5. Deletion of the second but not the first PDZ domain in NHERF2 abrogates the stimulating effect of SGK1/NHERF2 on TRPV5 activity and abundance at the plasma membrane. Thus, these data indicate that the C-terminal tail of TRPV5 interacts with the second PDZ domain of NHERF2 and this interaction is required for TRPV5 stabilization at or TRPV5 targeting to the plasma membrane (Palmada et al. 2005).

Modulation by Ca^{2+} signaling and calmodulin binding

Calmodulin (CaM) controls many Ca^{2+}-dependent cellular processes and is an important modulator of various types of ion channels. Several studies have been conducted to dissect specific CaM-binding domains (CaMBD) and determine their function for regulation of TRP channel activity (for recent review, see Zhu 2005). The first indication of functional interaction between CaM and TRP channels comes from a study devoted to isolation of CaM-binding proteins in *Drosophila* (Phillips et al. 1992). This study resulted in isolation of TRPL, a close homologue of *Drosophila* TRP. CaM-binding assays showed that TRPL has two CaMBDs in the C terminus, CaMBD1 (anino acids 710–725) and CaMBD2 (amino acids 859–871) (Phillips et al. 1992). CaMBD1 binds calmodulin in a Ca^{2+}-dependent way, while binding of CaM to CaMBD2 is Ca^{2+}-independent. Interaction between CaMBD1 and CaM is regulated by phosphorylation of two serine residues, S^{721} and S^{722} (Trost et al. 1999; Warr and Kelly 1996). Phosporylation of S^{721} by protein kinase A (PKA) abolishes the CaM binding, whereas phosporylation of the adjacent S^{722} by PKC results in modulation of phosphorylation by PKA.

Binding studies performed on TRPC3 revealed that the inositol 1,4,5-triphosphate (IP_3) receptor (IP_3R) and CaM interact directly with so-called CaM/IP_3R binding region (CIBR) at the C terminus of the channel (Boulay et al. 1999; Zhang et al. 2001). Interaction of CaM with TRPC3 has an inhibitory effect on the TRPC3 channel function. This inhibition is reversed in the presence of IP_3R, which competes for the binding to the CIRB region, resulting in displacement of CaM from the CIRB domain and activation of the channel (Zhang et al. 2001). Interestingly, TRPC3 mutants lacking the CIBR domain are predominantly localized in intracellular compartments, suggesting that CaM/ IP_3R binding can be involved

in trafficking of the channel to the plasma membrane (Wedel et al. 2003). By sequence homology analysis, the CIRB domain has been identified in all TRPC channels. However, the sensitivity and responses to CaM and IP_3Rs vary between different TRPC channels (Tang et al. 2001). In TRPC4, interaction with CaM also depends on two regions in the C terminus between residues 688–759 and 786–848, which bind CaM in Ca^{2+}-dependent manner (Trost et al. 2001). The C terminus of TRPC1 can interact with CaM in the two regions localized between animo acids 719–749 (CaMBD1) and 756–793 (CaMBD2) (Singh et al. 2002). Deletion of CaMBD1 region did not alter either thapsigargin-stimulated increase of the intracellular calcium level ($[Ca^{2+}]_i$) or Ca^{2+}-dependent feedback inhibition of the store operated calcium entry (SOCE). On the other hand, deletion of CaMBD2 of TRPC1 increases SOCE and decreases Ca^{2+}-dependent inactivation of the channel (Singh et al. 2002). Interestingly, it has been shown that the adaptor protein Homer facilitates a physical TRPC1–IP_3R association and is required for the TRP channel to respond to signals. The TRPC1-Homer–IP_3R complex is dynamic and its disassembly parallels TRPC1 channel activation (Yuan et al. 2003).

CaM-dependent regulation of the channel activity has also been assessed for several TRPV channels. TRPV1 exhibits two CaM binding sites. Disruption of CaMBD in the C terminus prevented TRPV1 desensitization (Numazaki et al. 2003), whereas binding of CaM to the N-terminal CaMBD decreases the capsaicin-activated currents (Rosenbaum et al. 2004). In TRPV4, CaM binds to a stretch of basic amino acids in the C terminus of the channel starting at position 814. Neutralization of positive charges in this region results in the loss of Ca^{2+}-dependent potentiation and of the spontaneous opening of TRPV4 in the absence of an agonist. The TRPV4 CaMBD also exhibits a consensus sequence for protein serine/threonine kinase phosphorylation, but mutations to these residues did not alter the Ca^{2+}-dependent potentiation (Strotmann et al. 2003).

In the case of TRPV5/6, two conserved CaMBDs have been identified in both the N and C termini (Lambers et al. 2004; Niemeyer et al. 2001). Interestingly, an additional CaM-binding site is present in the transmembrane region of TRPV6 (Lambers et al. 2004). The C-terminal CaMBD of TRPV6 overlaps with a consensus sequence for protein kinase C (PKC) phosphorylation. PKC-dependent phosphorylation of the site alters CaM binding and delays channel inactivation (Niemeyer et al. 2001). Co-expression of TRPV6 together with a CaM variant in which all four Ca^{2+}-binding sites (CaM_{1234}) are mutated significantly reduces inward Ca^{2+} currents upon hyperpolarization. No such effect can be observed for TRPV5-expressing cells (Lambers et al. 2004). Remarkably, Ca^{2+}-dependent inactivation of TRPV5 and TRPV6 are dramatically different; the initial inactivation of TRPV6 is much faster than that of TRPV5. Mutagenesis studies in TRPV6 show that residues L^{409}, V^{411}, and T^{412} in the intracellular loop located between TM2 and TM3 are responsible for the fast inactivation behavior of this channel (Nilius et al. 2002). In contrast, Ca^{2+}-dependent inactivation of TRPV5 is determined by two domains in the C terminus (Nilius et al. 2003b).

More recently, Nilius et al. found that overexpression of the CaM_{1234} mutant dramatically reduced TRPM4 activation (Nilius et al. 2005b). In vitro binding assays identified five short regions, two at the N terminus and three at the C terminus of TRPM4, which interact with CaM in a Ca^{2+}-dependent manner. Under Ca^{2+}-free conditions, four TRPM4 fragments display no binding and one shows weak binding to CaM. However, all CaM-binding fragments associate much more strongly with CaM in the presence than in the absence of Ca^{2+}. Interestingly, these CaM-binding sites appear to be multifunctional, as deletions of the C-terminal but not the N-terminal sites affected the Ca^{2+} sensitivity of TRPM4. Thus, all these data suggest that CaM binding to the C-terminal sites is vital for Ca^{2+} sensitivity of TRPM4 in the physiological range of intracellular Ca^{2+} concentrations (Nilius et al. 2005b).

Modulation by phosphorylation

Phosphorylation by protein kinases is a recurring and reversible post-translational modification that can regulate properties of ion channels. Studies of phorbolester- and Ca^{2+}-dependent protein phosphorylation in *Drosophila* demonstrate that, apart from the PDZ domain protein INAD, the TRP channel is a substrate of eye-specific PKC in isolated signaling complexes. This mechanism can be a part of a negative feedback loop that regulates Ca^{2+} influx through the channel (Huber et al. 1998).

TRPC3 can be phosphorylated by cGMP-dependent protein kinase G (PKG). Mutations at two consensus PKG phosphorylation sites, T^{11} and S^{263}, markedly reduce the inhibitory effect of cGMP on TRPC3-mediated store-operated Ca^{2+} influx. Treatment with PKG inhibitors had a similar effect (Kwan et al. 2004). More recently, it has been shown that TRPC3 is negatively regulated by PKC-dependent phosphorylation of S^{712}, a residue that is conserved in all mammalian TRPC channels. This mechanism is mediated by PLC-generated diacylglycerol, which serves both as a signal for TRPC3 activation and as a signal for negative feedback via PKC-mediated phosphorylation (Trebak et al. 2005).

Relatively limited data are available for other TRPCs. It has been shown that TRPC6 is directly phosphorylated by Src family protein-tyrosine kinases (PTKs) and this mechanism regulates TRPC6 channel activity (Hisatsune et al. 2004). TRPC5 can be phosphorylated by PKC. PKC inhibitors prevent TRPC5 desensitization after activation by G protein-coupled receptor, and the mutation of T^{972} to alanine dramatically slows this desensitization process. Thus, these results strongly suggest that desensitization of TRPC5 occurs via PKC-dependent phosphorylation of T^{972} (Zhu et al. 2005).

The functional role of phosphorylation on TRP channel function is probably best described in TRPV1. Early work on capsaicin and heat activation of TRPV1 revealed that this channel is a target for PKC-dependent phosphorylation (Chuang et al. 2001; Crandall et al. 2002; Hu et al. 2002; Numazaki et al. 2002; Premkumar and Ahern 2000; Tominaga et al. 2001; Vellani et al. 2001; Zhou et al. 2001b). Mutation of S^{800} to alanine significantly reduces phorbol 12-myristate 13-acetate (PMA)-induced enhancement of capsaicin-evoked currents and the direct activation of TRPV1 by PMA. In contrast, mutation of S^{502} to alanine reduces PMA enhancement of capsaicin-evoked currents with no effect on direct activation of TRPV1 by PMA, whereas mutation of T^{704} to alanine does not affect PMA enhancement of capsaicin-evoked currents but dramatically reduces direct activation of the channel by PMA. These results suggest that PKC-mediated phosphorylation modulates TRPV1 but does not directly gate the channel (Bhave et al. 2003; Numazaki et al. 2002; Vlachova et al. 2003). More recently, experiments conducted on a C-terminal truncated TRPV1 channel suggest that the distal C terminus of TRPV1 has an inhibitory effect on PKC phosphorylation-induced potentiation of the TRPV1 channel (Liu et al. 2004; Vlachova et al. 2003). TRPV1 can also be subjected for PKA-dependent phosphorylation. PKA-dependent phosphorylation of the N-terminal S^{116} interferes with the desensitization capsaicin-evoked whole cell currents (Bhave et al. 2002). Two other PKA phosphorylation sites in the C terminus, S^{774} and S^{820}, are also involved in regulation of TRPV1 channel desensitization (Mohapatra and Nau 2003). In contrast to TRPV1, a specific tyrosine residue localized in the first ankyrin motif of TRPV4 is phosphorylated upon hypotonic stress. This swelling-induced phosphorylation at Y^{253} is mediated via a member of Src family PTKs, the Lyn kinase, demonstrating that TRPV4 can be regulated by tyrosine phosphorylation (Xu et al. 2003). However, this mechanism of activation seems to be controversial since in a more recent study it has been shown that mutation of Y^{253} to phenylalanine does not affect hypotonic-induced activation of TRPV4 (Vriens et al. 2004b).

In the TRPMs, the effects of channel phosphorylation have only been described for TRPM4 and TRPM7. The Ca^{2+} sensitivity of TRPM4 is modulated by PKC-dependent phosphorylation. In the presence of ATP, PMA sensitizes Ca^{2+}-dependent activation of TRPM4. This effect is abolished when either of the two C-terminal serines, S^{1152} and S^{1145}, which are predicted to have the highest probability for PKC phosphorylation, are mutated (Nilius et al. 2005b). Mutation of these two serines to glutamates to mimic the phosphorylated state of the channel results in a delayed deactivation of TRPM4 and shifts the activation curves toward more negative voltages (Nilius et al. 2005c).

An interesting feature of TRPM7 is the presence in its C terminus of an atypical protein-kinase domain, the so-called phospholipase C interacting kinase (PLIK) domain (Runnels et al. 2001). The crystal structure of this protein kinase domain has been determined. In its catalytic core, it shows unexpected similarity to eukaryotic α-kinases (Yamaguchi et al. 2001). It has been shown that TRPM7 activity can be up- and down-regulated via the PLIK domain in a cAMP- and PKA-dependent manner (Takezawa et al. 2004). However, the importance of the PLIK domain for the TRPM7 channel function is still controversial. Inactivation of PLIK kinase activity by site-directed mutagenesis and/or changes in intracellular ATP indicated that the endogenous kinase activity is essential for channel function (Runnels et al. 2001).

Deletion of the region that comprises the kinase domain resulted in a functional channel with increased sensitivity to Mg^{2+} and MgATP. These data suggest that the structural kinase domain alters the sensitivity of TRPM7 to extracellular Mg^{2+} (Schmitz et al. 2003). More recently, it was shown that the PLIK domain autophosphorylates TRPM7 at serine residues, S^{1511} and S^{1567}. Mutation of these two sites or of the catalytic site that abolished kinase activity (kinase-dead mutants) did not affect the channel function and inhibition by internal Mg^{2+} but abolished the kinase activity. Divalent cations such as Mg^{2+}, Zn^{2+}, and Ca^{2+} inhibit the channel activity. In contrast, the kinase activity is enhanced by Mg^{2+}, decreased by Zn^{2+} and in the case of Ca^{2+} no effects have been observed (Matsushita et al. 2005). In contrast to Schmitz et al. (2003), the authors of this latest study did not see functional expression of TRPM7 lacking the full kinase domain. Therefore, they suggested that neither current activity nor regulation by internal Mg^{2+} is affected by kinase activity or autophosphorylation, but that the kinase domain may play a structural role in channel assembly or subcellular localization (Matsushita et al. 2005).

Modulation by PIP_2 and possible PIP_2 binding sites

The first example of PIP_2-dependent modulation of a TRP channel was described for TRPV1, whose function is inhibited by PIP_2. Hydrolysis of PIP_2 by stimulation of PLC reverses the TRPV1 inhibition (Chuang et al. 2001). The molecular determinant for the PIP_2 interaction in TRPV1 channels is localized in the C terminus of TRPV1 between amino acids 777–820 (Prescott and Julius 2003). Similar effects are also observed for *Drosophila* TRPL channels. In excised inside-out patches, the spontaneous TRPL channel activity is strongly reduced upon application of PIP_2. Surprisingly, this effect is not observed in all patches. The reasons why PIP_2 is unable to inhibit TRPL in all patches is not known, but it is possible that there could be a state dependence of the TRPL channel necessary for the effect of PIP_2, or that some of the excised patches lack a specific protein (Estacion et al. 2001).

As already mentioned in the section entitled “Modulation by phosphorylation”, TRPM5, TRPV5, and TRPM8 are activated by interaction with PIP_2 to specific consensus residues in

the TRP domain (Liu et al. 2005; Liu and Liman 2003; Rohacs et al. 2005). Similar effects of PIP_2 have also been shown for activation of the TRPM7 channel, which becomes inactive upon stimulation of PLC activation and PIP_2 hydrolysis (Runnels et al. 2002). Recovery of carbachol induced TRPM7 current inhibition is accelerated after wash out in the presence of PIP_2. Furthermore, application of PIP_2 to inside-out patches after rundown results in a full restoration of TRPM7 single-channel activity (Pedersen et al. 2005; Runnels et al. 2002). For TRPM5, it has been shown that PIP_2 reverses the desensitization of the channel caused by a sustained exposure to Ca^{2+}, resulting in a partial recovery of the channel activity (Liu and Liman 2003). Interestingly, the closest TRPM5 homologue, TRPM4, contains a putative PIP_2-binding domain, which shares homology with pleckstrin domains (Nilius et al. 2006). Mutation of this putative PIP_2-binding domain of TRPM4 prevents activation of the channel by PIP_2 and decavandate, a compound with six negative charges, which may mimic the PIP_2 action (Nilius et al. 2004a, Nilius et al. 2006). In TRPM8, channel activation causes an influx of Ca^{2+}, which activates Ca^{2+}-sensitive PLC-dependent hydrolysis of PIP_2, resulting in closure or desensitization of the channel (Liu et al. 2005).

Endogenous enzymatic activities

The presence of a full enzyme in the C terminus is not only found in TRPM7 (see "Modulation by phosphorylation"). A similar α-kinase domain is also found in TRPM6, the closest homologue of TRPM7 (Chubanov et al. 2004; Schlingmann et al. 2002). Another TRPM member, TRPM2, contains a Nudix hydrolase domain in its C terminus, which functions as an ADP-ribose (ADPR) pyrophosphatase (Kuhn et al. 2005; Perraud et al. 2001; for review see Perraud et al. 2003). The TRPM2 Nudix domain shares 39% identity with NUDT9, a human ADPR pyrophosphatase. A characteristic feature of many members of the Nudix enzyme family is the presence of the conserved Nudix box, $GX_5EX_7REuXEEXu$ (X any amino acid residue and *u* a large hydrophobic residue). In TRPM2, some of the key positions in Nudix box are altered. Introduction of these different amino acids into the NUDT9 causes a strong decrease in the ADPR activity, similar to that obtained for TRPM2. The crystallographic data show that unlike its closest functional homologue, homodimeric *Escherichia coli* ADPRase, NUDT9 is active as a monomer with the substrate binding site located in a cleft between the N-terminal and the C-terminal catalytic domain (Shen et al. 2003).

It has been shown that ADPR activates the TRPM2 channel, suggesting that the enzymatic activity of the Nudix domain is an essential component of the gating mechanism of the channel (Perraud et al. 2001). The C-terminal splice variant of TRPM2, which contains a deletion of 34 amino acids in the region (between amino acids 1292–1325) distant from the Nudix box (DeltaC-strech), can still be activated by H_2O_2 but does not respond to ADPR (Wehage et al. 2002). Mutants lacking 19, 25, and 29 amino acid residues in the N-terminal part or having substitutions of amino acid residues in the remaining C-terminal part of the DeltaC stretch displays typical ADPR-induced currents. However, deletion or substitution of the amino acid residue N^{1326} immediately downstream of the DeltaC stretch abrogates ADPR gating. These data suggest that amino acid residues in the DeltaC stretch are not directly involved in ADPR gating but may act as a spacer segment stabilizing a conformation necessary for the essential N^{1326} residue to interact with other channel regions. Interestingly, prolonged binding rather than degradation of ADPR is required for the modulation of TRPM2 function, since enhancement of Nudix box activity abolishes the ADPR gating of the channel (Kuhn and Luckhoff 2004).

More recently, it has been shown by structure-guided mutagenesis that TRPM2 gating by ADPR and both oxidative and nitrosative stresses requires an intact ADPR binding cleft in the C-terminal NUDT7 domain (for a recent review, see Kuhn et al. 2005). The oxidative/nitrosative stress-induced TRPM2 gating can be inhibited by blocking ADPR production and by suppressing ADPR accumulation by cytosolic or mitochondrial overexpression of an enzyme that specifically hydrolyzes ADPR (Perraud et al. 2005).

Concluding remarks

In this review we have given an overview of the most recent data on the structure–function relationship of TRP channels. A broad range of thus far identified structural domains and motifs strongly emphasizes the diversity of functions and regulatory mechanisms in the TRP superfamily. However, it has to be stressed that despite the rapid progress made in the last few years, a detailed view on the role of particular domains in regulation of the channel function is still elusive. Continuation of scientific efforts will be required to further clarify the structural basis for the functioning of the fascinating superfamily of TRP channels.

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