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Molecular Pathogenesis of MODYs

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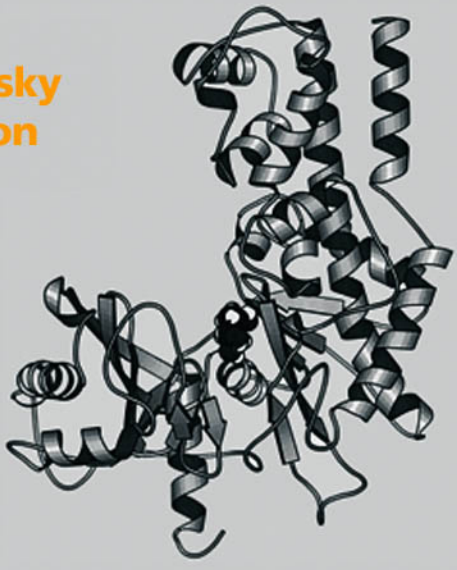

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Volume Editors *F.M. Matschinsky*, Philadelphia, Pa.
M.A. Magnuson, Nashville, Tenn.

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Franz M. Matschinsky, MD

Department of Biochemistry and Biophysics and
Diabetes Research Center, University of Pennsylvania
Medical Center, Philadelphia, Pa., USA

Mark A. Magnuson, MD

Department of Molecular Physiology and Biophysics
Vanderbilt University School of Medicine
Nashville, Tenn., USA

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Contents

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Preface

On November 6–8, 1998, the American Diabetes Association sponsored a Research Symposium titled 'MODYs as Paradigm of the Pathogenesis of NIDDM' in Scottsdale, Arizona. We are pleased to present the proceedings of this symposium in this volume (No. 15) of '*Frontiers in Diabetes*'. Written accounts of 18 of the 20 oral presentations made by invited participants are presented in the order that they were delivered in Scottsdale.

Planning for this symposium began in 1996 and was initially focused on glucokinase-linked diabetes mellitus (MODY-2). However, thanks to the advice of the consultants for the meeting (Drs. M. German, D. Granner, C. Newgard, A. Permutt, and K. Polonsky), and greatly influenced by the stunning concurrent advance in the MODY field, we broadened the scope as the planning progressed. The reference to NIDDM in the symposium title served as a charge to participants to connect the evolving knowledge about MODY to the pathogenesis of NIDDM in general. This was, of course, a very difficult task that was only achieved peripherally but one that will continue to drive this field.

We hope that this publication will rekindle the excitement we all felt while we listened to and discussed the remarkable progress that has been made in understanding the molecular pathogenesis of the MODYs. We also hope that this volume will be of benefit to those who did not have the opportunity to attend the meeting. In time, we hope that this volume will be viewed as a notable milestone on the way to a fuller understanding of the molecular basis of type 2 diabetes mellitus. We have made sizeable strides in understanding the molecular basis of this disease by studying the MODYs, but much more progress remains to be achieved.

Lastly, we thank Ms. Linda Cann and Ms. Shirley Ash from the American Diabetes Association for selecting the attractive location and providing superb organizational support. The meeting would not have been a success without their efforts. We also thank Eli Lily, Pharmacia-Upjohn, Pfizer and Mrs. Elke Matschinsky for helping the organizers to cover the publication costs for the volume.

Franz M. Matschinsky
Mark A. Magnuson

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Natural History, Genetics and Pathogenesis of HNF-4α/MODY1

A 40-Year Prospective Study of the RW Pedigree

Stefan S. Fajans[a], *Graeme I. Bell*[c, d], *William H. Herman*[a], *Kenneth S. Polonsky*[d], *Jeffery B. Halter*[a, b]

[a] Department of Internal Medicine, University of Michigan Medical Center;
[b] Department of Veterans Affairs Medical Center, Ann Arbor, Mich.;
[c] Departments of Biochemistry and Molecular Biology and Human Genetics and Howard Hughes Medical Institute, and
[d] The Department of Medicine, The University of Chicago, Ill., USA

History and Definition

In 1960 we first reported in prospective studies that in first-degree relatives of type 2 diabetic patients mild, asymptomatic diabetes may occur in nonobese children, adolescents, and young adults and that their diabetic glucose tolerance and fasting hyperglycemia may be improved or normalized by sulfonylurea therapy, currently for up to 4 decades [1–3]. In 1965, we first used the term maturity-onset type diabetes of childhood or of young people for this type of diabetes and emphasized its strong familial association [4]. In 1974 Tattersall reported on a mild form of diabetes in three families from King's College Hospital in London and recognized that their inheritance conformed with an autosomal dominant pattern [5]. In 1975 Tattersall and Fajans differentiated between the inheritance of diabetes in 35 families with type 1 diabetes and that of 24 Michigan families with maturity-onset diabetes of the young, confirmed autosomal dominant inheritance for the latter, and first used the abbreviation or acronym MODY [6]. Earlier, Cammidge had suggested the existence of a mild form of familial diabetes with dominant inheritance based on the finding of glucosuria; blood glucose levels were not reported [7].

Maturity-onset diabetes of the young (MODY) was initially defined as a subtype of type 2 diabetes characterized by onset at a young age, usually under

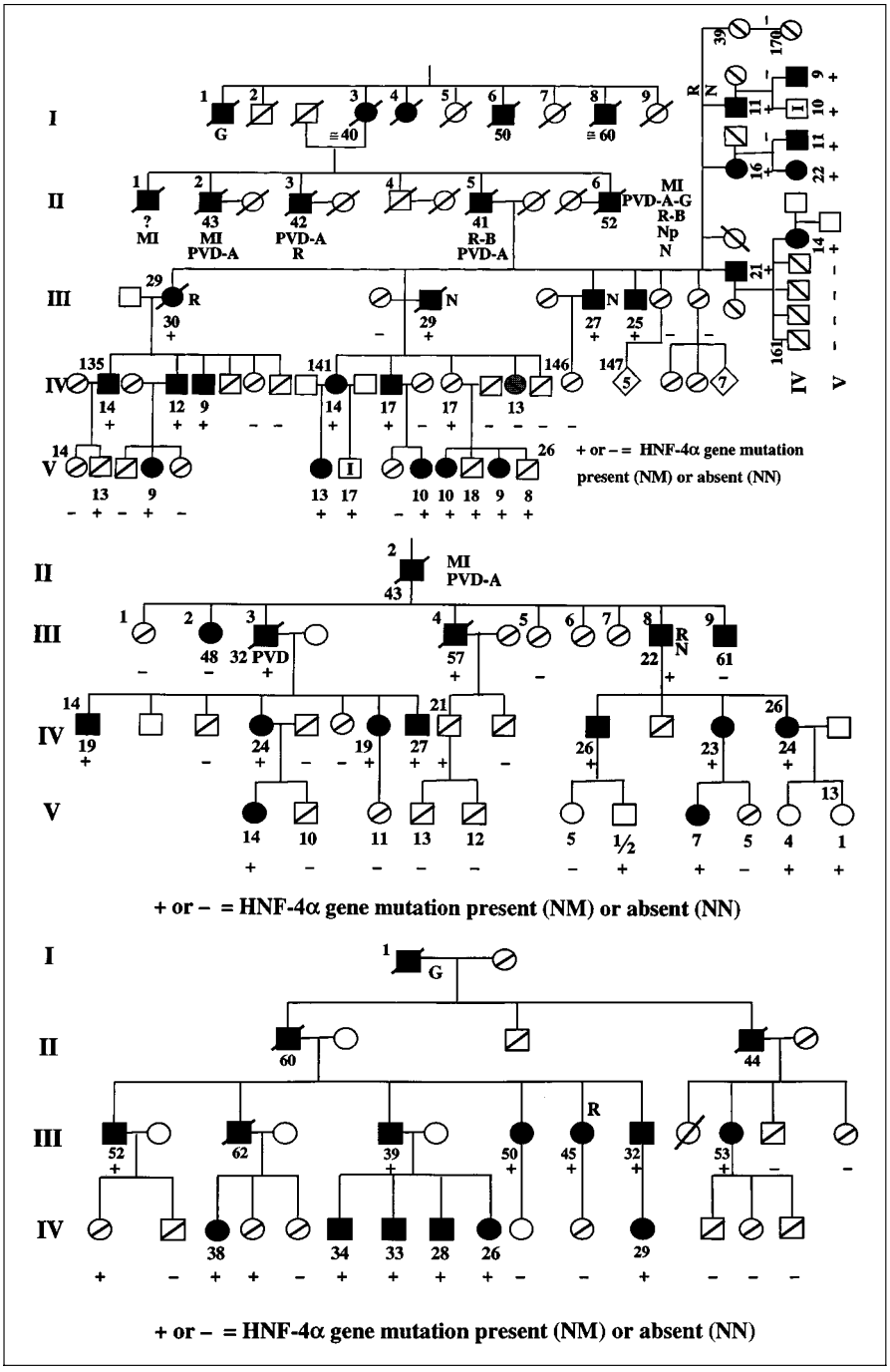
age 25 years, plus autosomal dominant inheritance [8–11]. It is now clear that MODY is a group of heterogenous monogenic disorders, all of which are characterized by nonketotic diabetes, autosomal dominant inheritance, onset usually before 25 years of age, and a primary defect in insulin secretion. MODY can result from heterozygous mutations in at least five different genes: the glycolytic enzyme, glucokinase/MODY2 [12] and the transcription factors, hepatocyte nuclear factor (HNF)-4α/MODY1 [13], HNF-1α/MODY3 [14], HNF-1β/MODY5 [15] and insulin promoter factor-1/MODY4 [16].

A primary defect in insulin secretion was added to the definition of MODY as decreased insulin responses to orally and intravenously administered glucose, or defective insulin secretion, have been demonstrated not only in prediabetic and diabetic members of the RW/MODY1 Pedigree [8, 10, 17, 18] which forms the substance of this report, but also in glucokinase/MODY2 [19, 20] and in prediabetic and diabetic MODY3 [21, 22]. The revised ADA classification of diabetes categorizes MODY under 'genetic defects of β-cell function' rather than under type 2 diabetes [23].

Phenotypic Expression and Natural History

Because of autosomal dominant inheritance and early age of onset it is possible to collect large multi-generational pedigrees with MODY, a unique feature of this type of diabetes. When MODY pedigrees are studied prospectively, much can be learned about the phenotypical expression and natural history of this type of diabetes. The largest and best studied MODY family

Fig. 1. Partial pedigree of the RW family. Offspring of II-5 (*a*) and II-2 (*b*) of the W branch and offspring of I-1 (*c*) of the R branch are shown. All subjects with diabetes have MODY or type 2 diabetes except IV-144 who has type 1 diabetes. (+) or (−) indicates HNF-4α gene mutation status: present (NM) or absent (NN). IV-143 (A) who carries the HNF-4α mutation is indicated as nondiabetic even though she had diabetic glucose tolerance tests at ages 17 and 40 years, respectively (see text). IV-168 (*a*) has had one diabetic GTT. The older son of III-4 (*b*) is nonpenetrant. In the R branch (*c*), generation IV, two females are indicated as nondiabetic but HNF-4α mutation-positive. Each had a normal fasting plasma glucose concentration but neither has been tested with a GTT. Numbers above male diabetic members (■), or female diabetic members (●), are number in generation and those below are ages at diagnosis. ◫ = Impaired glucose tolerance; ∅ = normal glucose tolerance; □ = reported normal and untested; ⍁ = deceased; ◈ = groups of siblings untested and of unspecified sex with number of individuals given. The abbreviations used for complications are: MI = Myocardial infarction; PVD = peripheral vascular disease; A = amputation; G = gangrene; N = neuropathy; Ang = angina pectoris; R = retinopathy; R-B = retinopathy and blindness; Np = nephropathy.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is the RW Pedigree, a Caucasian family of German/East Prussian ancestry, which has been followed prospectively for over 40 years by one of us (SSF). It consists of 445 members in seven generations, including 75 diabetic subjects in the last 5 generations. MODY in the RW family is due to inheritance of a nonsense mutation, Q268X, in the HNF-4α/MODY1 gene [13].

The propositus, II-5 (W branch), offspring of a diabetic mother (I-3), had diabetes diagnosed at the age of 41 years (fig. 1a). He was blind from diabetic retinopathy at the age of 61 years and had an amputation for peripheral vascular disease. He had five brothers, four of whom had diabetes. Macrovascular disease including myocardial infarctions and peripheral vascular disease with gangrene and amputations, microvascular disease including retinopathy and blindness, and neuropathy are complications resulting from MODY in the RW pedigree (fig. 1a). The diabetic mother and four other diabetic subjects in this sibship of 9 constitute diabetic generation I. In the two preceding generations there was no known diabetes. In 1958, the eleven nonobese, apparently healthy and asymptomatic offspring of II-5 were recruited for routine blood glucose testing. Seven of the eleven, ranging from age 30 to 11 years, were found to have abnormal glucose metabolism [24]. The older three had fasting hyperglycemia (mean 257 mg/dl), three others had diabetic glucose tolerance tests without diagnostic fasting hyperglycemia and one had impaired glucose tolerance (IGT). With follow-up, these 4 have also developed fasting hyperglycemia. Two have been treated with insulin because of eventual unresponsiveness to sulfonylurea drugs, and five have been treated successfully with sulfonylurea drugs up to the present. Subsequently, by routine blood glucose testing diabetes was diagnosed in 11 of the 21 members of generation IV who are offspring of diabetic subjects of generation III, and up to the present time in five members of generation V of this branch. None of them are obese. Ages of first testing and diagnosis were 9–14 years in twelve of these patients with diabetes. Patient IV-144 has typical type 1 diabetes. She does not have the HNF-4α mutation. All the MODY diabetic subjects, including three 9-year-old prepubutal boys in this branch, are HNF-4α mutation-positive as are a few nondiabetic children including a 1-year-old child, offspring of IV-165. IV–143, age 41, who carries the Q268X nonsense mutation is shown as being nondiabetic in fig. 1a. Having been tested with oral glucose tolerance tests (GTT) from age 16 years on, she had one diabetic GTT at age 17 and a second one at age 40 years while the remainder of the at least yearly GTTs during this 23-year period have been within normal limits. She has low insulin responses to orally and intravenously administered glucose. She is lean and very active and showed increase sensitivity to insulin (S_I) during a frequently sampled intravenous glucose tolerance test [17]. During low dose glucose infusions she became markedly hyperglycemic.

Among offspring of II-2, three of the five diabetic members (III-3, III-4, III-8) have MODY as do nine subjects in generation IV and V (fig. 1b). The youngest, 7 years old at diagnosis, had fasting hyperglycemia of 203 mg/dl. We have genotyped children ranging from 6 months and one year to 5 years of age at their parents' request to ascertain whether they have inherited the at-risk diabetes mutation. Three of five of these children carry the mutation. Two of the five diabetic members of generation III (III-2, III-9), are obese, are hyperinsulinemic, while MODY subjects are generally hypoinsulinemic (see below), and were diagnosed with diabetes at age 48 and 61 years, respectively. They and their offspring are HNF-4α mutation-negative. Thus, offspring of II-5 and some of II-2 exhibit the classic pattern of autosomal dominant inheritance characteristic of MODY whereas the offspring of III-2 and III-9 and their offspring, as well as the offspring of II-3 and II-6 do not. Thus clinical and molecular genetic studies indicate the existence of heterogeneity of inheritance and type of diabetes in this large pedigree.

I-1 had three offspring, two of whom had diagnosed diabetes (R branch, generations II, fig. 1c). Among their 10 offspring in generation III, seven have or have had diagnosed diabetes. Of fourteen tested subjects in generation IV, who are offspring of a diabetic parent, 6 have diabetes. Thus, diabetes is inherited in the autosomal dominant pattern of MODY in the R branch as well. None were tested and diagnosed before the age of 26 years.

MODY is usually asymptomatic in young people, although some patients may have symptoms, particularly if stressed by an infection. Unless searched out by prospective testing in a young person, because of family history of type 2 diabetes in two or more generations, a clinical diagnosis of diabetes is frequently not made in many members of such families until middle or late adult life. Thus, age at diagnosis cannot be equated to age of onset of hyperglycemia. As demonstrated in the RW pedigree by repeated testing in younger members, there may be a variable rate of progression from normal or nondiagnostic glucose tolerance tests to IGT, from IGT to diabetic glucose tolerance with normal fasting plasma glucose levels, and very slow or rapid progression to fasting hyperglycemia (0.5–27 years). Severity of carbohydrate intolerance may fluctuate for many years, particularly in patients with mild abnormalities, before the onset of persistent fasting hyperglycemia which eventually occurs in approximately 95% of diabetic members. In contrast, other individuals with MODY from this pedigree may have fasting hyperglycemia at diagnosis at an early age and rapid progression in severity, even though they are asymptomatic. Table 1 gives nine examples of fasting hyperglycemia diagnosed between ages of 7 and 14 years at first testing. Five of these nine subjects progressed to insulin requirement within 3 to 25 years.

Table 1. Examples of fasting hyperglycemia at diagnosis (Dx) in children and adolescents of RW/MODY1 pedigree

Subject Gen.-No.	Age at DX Yrs.	FPG (mg/dl)	HbA1 (%)	Current treatment Sulfonylurea or insulin (yrs after Dx)
III-38	11	125		Insulin (25)
IV-135	14	167		Insulin (25)
IV-136	12	174		Insulin (23)
IV-165	14	192	7.6	Sulfonylurea (13)
IV-169	9	146	8.6	Insulin (4)
V-3	14	200	10.3	Sulfonylurea (10)
V-10	7	203	9.7	Sulfonylurea (2)
V-19	13	147	7.5	Insulin (3)
V-22	10	376 (pp)	9.3	Sulfonylurea (9)

pp = Postprandial.

Fasting hyperglycemia, even at a young age of 7 to 14 years, may be responsive to diet plus oral agents for a few years to up to four decades. In paired tests, one on sulfonylureas and the second 3 weeks off sulfonylureas, glucose-induced insulin secretion during GTTs showed an average increase of 68% which persisted for decades in diabetic patients who remained responsive [3]. In the majority of patients however, glucose-induced insulin secretion declined over time to a varying degree, at a rate of 1–4% per year [3]. Approximately 30% of MODY1 patients of the RW pedigree have become unresponsive to maximal dosages of sulfonylureas after 3 to 25 years and they require treatment with insulin to normalize fasting and postprandial hyperglycemia. At this stage some of these patients have very low fasting and nutrient-stimulated insulin and C-peptide levels and an unstable type of diabetes resembling that seen in type 1 diabetes. Typical microangiopathic, macroangiopathic and neuropathic complications may occur in similar frequency to those seen in patients with classical type 2 diabetes, if poorly controlled.

Molecular Genetics

In defining the molecular genetic basis of MODY linkage analysis has been successful in view of autosomal dominant inheritance, availability of large multigenerational kindreds and an easily recognizable phenotype. For

the RW pedigrees, Bell et al. reported linkage of diabetes with the adenosine deaminase (ADA) gene on chromosome 20 in 1991 [25] and shortly thereafter Bowden et al. found linkage with the anonymous loci D20S16 and D20S17 [26]. In 1996, a mutation (Q268X) was found in the transcription factor hepatocyte nuclear factor-4α by Yamagata et al. [13], indicating that this was the long sought after MODY1 gene.

Islet-Cell Function and Insulin Action

The majority of MODY1 subjects who are offspring of II-2 and II-5 have a delayed and subnormal insulin secretory response to orally administered glucose, suggesting strongly an impairment of β-cell function [8–11]. The low insulin secretory response to glucose may occur from childhood on and before glucose intolerance appears. This is demonstrated by insulin levels during the oral glucose tolerance test in nondiabetic subjects of the RW pedigree who are HNF-4α mutation-positive or -negative (fig. 2). The nondiabetic HNF-4α mutation-negative subjects had a mean insulin response to administration of glucose which was almost identical to that previously reported in 150 control subjects without a family history of diabetes [27]. In contrast, nondiabetic subjects who are mutation-positive, i.e. prediabetic subjects, had a decreased insulin response to glucose, maximum levels at 1 hour being approximately one-half of that of the mutation-negative subjects. Subjects with IGT had a similar or slightly lower insulin response to glucose. In MODY1 diabetic subjects with fasting plasma glucose levels below 140 mg/dl there was a further decline in the insulin response to glucose with additional progressive decreases in diabetic subjects with fasting plasma glucose concentrations between 140 and 200 and greater than 200 mg/dl (fig. 2). The defective insulin secretory response, although moderate in magnitude, appears to be a manifestation of the basic genetic defect and the major underlying pathogenetic factor that leads to abnormal glucose levels and diabetes although additional superimposed environmental facts undoubtedly contribute. Such factors include a decreasing physiological insulin sensitivity, without the normally associated increase in insulin secretion, that occurs with increasing age, growth and increasing body mass, as well as puberty, when subjects progress from childhood, to adolescence to young adult age [10]. Pathological insulin resistance does not appear to be a contributing factor because of normal sensitivity to intravenously administered insulin [17] and because of the usually low insulin requirement in insulin-treated diabetic members of the RW pedigree.

Since chronic hyperglycemia has been reported to be associated with both a decrease in insulin sensitivity as well as an impairment of β-cell

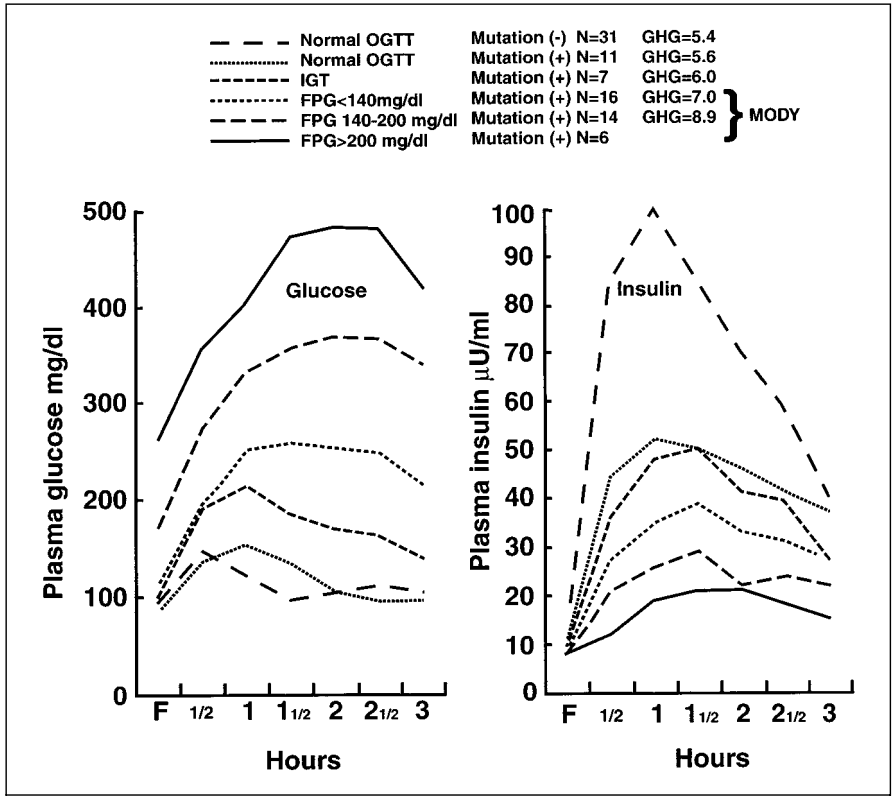

Fig. 2. Plasma concentrations of glucose and insulin during oral glucose tolerance tests (1.75 gm/kg body weight) in groups of subjects of the RW pedigree. Mutation (−) or (+) = HNF-4α mutation absent or present.

function, the question of which is the primary genetic defect in MODY, or any type of diabetes, is very difficult to address in diabetic patients [28]. Thus, more recently we have carried out a series of studies of genetically at risk but presently nondiabetic, i.e. prediabetic, subjects in order to define the earliest abnormalities in insulin action and secretion using a number of different protocols. In the first study, insulin action and insulin secretion were assessed with the frequently sampled intravenous glucose tolerance test [17]. Insulin secretion was further assessed during constant low dose glucose infusion by deconvolution of plasma C-peptide concentrations and by pulse analysis [17]. Six nondiabetic mutation-negative and five nondiabetic mutation-positive members of the RW pedigree were studied as were four mildly diabetic mutation-positive subjects. Nonrelated young healthy subjects served

as a further comparison group. The nondiabetic mutation-positive group had unimpaired acute insulin responses to intravenous glucose and tolbutamide. If anything, the insulin levels were lower in the mutation-positive, than the mutation-negative group. On the other hand, the glucose levels during the tests were identical. This indicates that there is no decrease in insulin sensitivity. However, during prolonged glucose infusion the nondiabetic mutation-positive group had decreased plasma C-peptide concentrations, reduced absolute amplitude of insulin secretory ultradian oscillations and a decreased insulin secretion rate. These responses to prolonged glucose infusions were similar to those observed in the diabetic group who in addition also had a decrease in the acute insulin response to intravenous glucose [17]. It was concluded that deranged and deficient insulin secretion, and not insulin resistance appears to be the genetic or primary abnormality that characterizes susceptibility to MODY1 and leads to diabetes [17]. Prolonged glucose infusion studies may reveal qualitative and quantitative defects in insulin secretion not identified by the acute insulin response to intravenous glucose. In diabetic MODY1 subjects, a decrease in insulin sensitivity, when present, is secondary to hyperglycemia.

Further definition of the insulin secretory defect in nondiabetic members of the RW pedigree and quantitative and qualitative differences in insulin secretory defects between prediabetic MODY1, MODY2 and prediabetic MODY3 subjects have been found by studies with a stepped glucose infusion protocol [18]. Increasing amounts of glucose from 1 to 8 mg/kg/min were infused each for 40-min periods. This was followed by glucose infusion for 42 hours at a rate of 4 to 6 mg/kg/min for glucose priming. Thereafter, the stepped glucose infusion protocol was repeated. For the RW pedigree, one half of the experiments were performed in the University of Michigan CRC and one half in the University of Chicago CRC [18]. Six mutation-negative subjects demonstrated a normal increase of insulin secretion rate with increasing plasma glucose concentrations. On the other hand, ten nondiabetic mutation-positive subjects, after giving a normal response up to 7 mM glucose concentration, showed a decreased response of the β-cell to glucose resulting in a downward shift in the glucose/insulin secretion rate dose-response curve. In the mutation-negative subjects, glucose priming by a low dose glucose infusion resulted in a shift of the curve to the left, i.e. there was an increase in β-cell sensitivity to glucose as seen in other control subjects. On the other hand, nondiabetic mutation-positive subjects showed failure of β-cell priming [18]. These results differ from those obtained in MODY 2 subjects with glucokinase mutations, who showed a major defect in the glucose/insulin dose response curve below 7 mM glucose and a continuing though decreased rise in insulin secretion with rising glucose concentrations [20]. Furthermore, MODY2 subjects showed

normal priming of insulin secretion by glucose [20]. As shown by an international group of investigators, including one of our Michigan MODY3 pedigrees, nondiabetic MODY 3 subjects showed a defect during the stepped glucose infusion similar to that previously shown for our MODY1 subjects [21]. However, in contrast to prediabetic MODY1 subjects who showed no glucose priming [18], prediabetic MODY3 subjects exhibited normal glucose priming [21]. The stepped glucose infusion protocol and the glucose priming procedure allow for a differentiation of prediabetic MODY1, MODY2, and prediabetic MODY3 subjects from each other.

We also ascertained the effect of the nonglucose secretagogue arginine on insulin and glucagon secretion and on glucose potentation of arginine-stimulated insulin secretion in three groups of subjects of the RW pedigree: (1) Seven nondiabetic subjects without HNF-4α mutations, (2) seven nondiabetic (prediabetic) subjects with HNF-4α mutations, and (3) four mildly diabetic subjects with HNF-4α mutations [29]. During a 25-minute arginine infusion the mutations-negative group showed a normal rise in insulin and C-peptide levels which was greatly enhanced when the arginine infusion was repeated during a glucose clamp at 11 mM glucose concentration. The nondiabetic mutation-positive group showed a greatly attenuated insulin and C-peptide response which was closer to that of the diabetic group than to that of the normal subjects both at basal glucose concentrations as well as during the hyperglycemia clamp [29]. Insulin secretion rate was significantly decreased in the nondiabetic and diabetic mutation-positive groups both at 6 and 11 mM glucose concentrations (fig. 3).

During the arginine infusion, plasma glucagon levels were also decreased in the mutation-positive groups. Areas under the curve for plasma glucagon during the arginine infusion were significantly decreased in the diabetic group (fig. 4) with the nondiabetic mutation-positive group having areas under the curve between the control and diabetic groups. Suppression of areas under the curve for plasma glucagon during the arginine infusion induced by the hyperglycemic clamp were less for the two mutation-positive groups than for the mutation-negative group (fig. 5). We concluded that both prediabetic and diabetic MODY1 subjects with Q268X mutation in the HNF-4α gene have a defect in insulin secretion in response to arginine as well as to glucose and have a defect in glucagon secretion in response to infused arginine. The defect in β-cell and α-cell secretion may be at a step in signal transduction common to both cell types or due to a decrease in β-cell and α-cell mass since HNF-α is a transcription factor involved in early pancreatic development and growth [29]. Further work is necessary to differentiate between these two possibilities, although at this time it appears that defective signal transduction is the more likely defect.

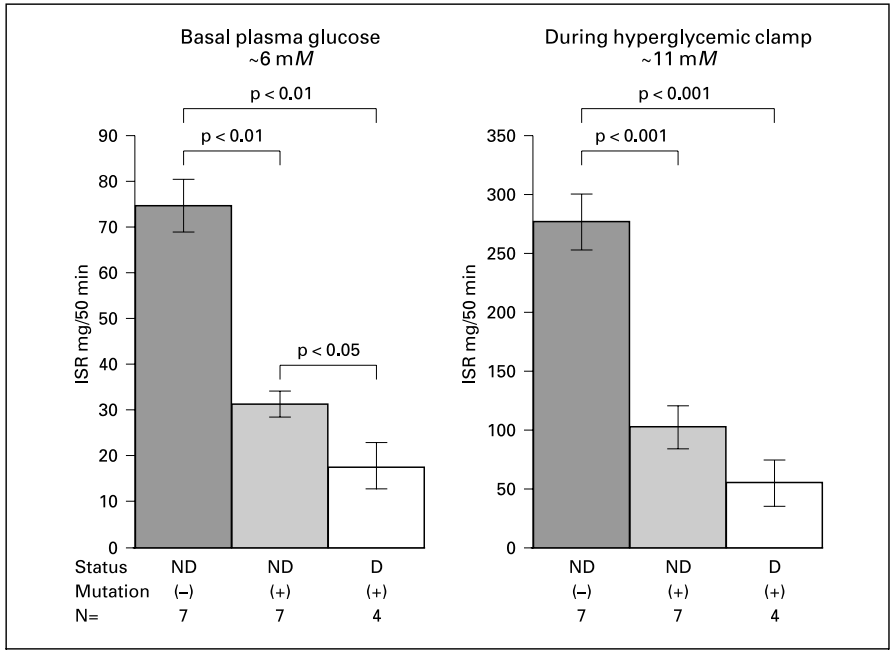

Fig. 3. Insulin secretion rates (ISR) by deconvolution of C-peptide during arginine infusion (10–60 minutes) in members of the RW pedigree [29]. (Note difference in scale for infusions at different plasma glucose concentration.) ND = Nondiabetic; D = diabetic; (−) = HNF-4α mutation absent; (+) = HNF-4α mutation present.

Potential Surrogate Markers

HNF-4α is believed to be essential in controlling transcription of many genes throughout life, particularly in the liver but also in the pancreas. Because of the known importance of HNF-4α for regulation of human clotting factor IX transcription [30] it was deemed to be of interest to examine plasma concentrations of factor IX by ELISA and one-stage clotting assays. Factor IX concentrations of 9 diabetic members of the RW pedigree were indistinguishable from those of control subjects (Brownlee, G.G., pers. commun.).

At least six other HNF-4α/MODY1 pedigrees have been identified worldwide [31–36]. In two of these pedigrees, the concentration of lipoprotein(a) were reported to be increased threefold in six subjects of one pedigree [31] and mildly elevated in three subjects of another [37]. This suggests that elevated levels of lipoprotein(a) [Lp(a)] might be a surrogate marker for HNF-4α/MODY1. In the RW/MODY1 pedigree 12 diabetic subjects had Lp(a)

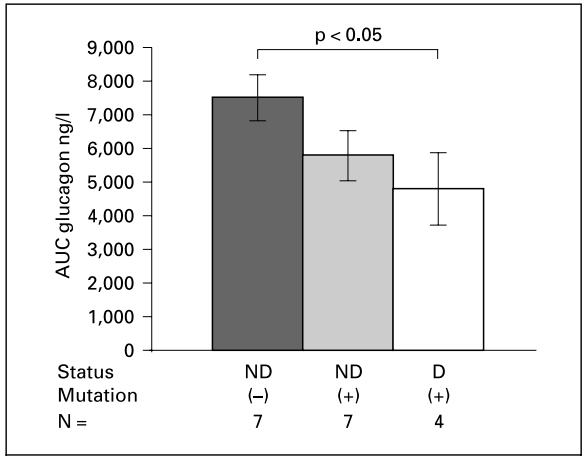

Fig. 4. Areas under the curve (AUC) for plasma glucagon during arginine infusion (10–60 minutes) at basal plasma glucose concentration in members or RW pedigree [29]. ND = Nondiabetic; D = diabetic; (−) = HNF-4α mutation absent; (+) = HNF-4α mutation present.

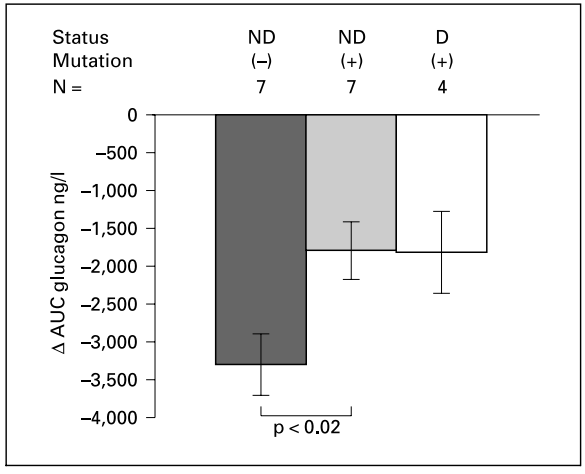

Fig. 5. Decreases in areas under the curve (AUC) for plasma glucagon during arginine infusion (10–60 minutes) induced by hyperglycemic clamp in members of the RW pedigree [29]. ND = Nondiabetic; D = diabetic; (−) = HNF-4α mutation absent; (+) = HNF-4α mutation present.

values (mean 12 mg/dl) which were similar to those of five nondiabetic mutation-positive subjects (mean 9 mg/dl) and not significantly different from those of six nondiabetic mutation-negative subjects (mean 27 mg/dl) (normal range 0–45mg/dl). The combined mutation-positive subjects (N = 17) were also not different from the mutation-negative subjects. Thus, Lp(a) concentrations are not elevated in nondiabetic or diabetic members of the MODY1/RW pedigree who carry the mutation in the HNF-4α gene.

Applications

Present knowledge allows clinical applications. Molecular genetic screening and diagnosis is possible in young subjects at risk for MODY and has important prognostic implications. Genetically susceptible MODY1 subjects can be counselled to have periodic evaluation of glucose tolerance beginning at 6 or 7 years of age. Early treatment to achieve normoglycemia beginning at the time of appearance of metabolic abnormalities should prevent vascular and neuropathic complications.

Finally, the molecular genetic and clinical studies of MODY can serve as a model for investigations of pathogeneses of more genetically complex forms of diabetes such as type 2 diabetes.

Acknowledgments

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Stefan S. Fajans, MD, Division of Endocrinology and Metabolism, 3920 Taubman Center,
Box 0354, University of Michigan Medical Center, Ann Arbor, MI 48109-0354 (USA)
Tel. +1 734 936 5504, Fax +1 734 936 9240

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Phenotype-Genotype Relationships in Maturity-Onset Diabetes of the Young

Andrew T. Hattersley, Sian Ellard, Maggie Shepherd,
Timothy M. Frayling, Mike Bulman, Liz Ballantyne, Susan Ayres

Department of Vascular Medicine (Diabetes Research), Postgraduate Medical School, Barrack Road, Exeter, UK

Introduction

The definition of the genetic basis of maturity-onset diabetes of the young (MODY) would not have occurred without the initial clinical observation recognizing a subgroup of early-onset non insulin-dependent diabetes characterized by dominant inheritance. In this way the outstanding clinical scientists such as Fajans, Tattersall and Lestredet greatly assisted the molecular geneticists. Now that the majority of the genetic heterogeneity of MODY has been defined, can the molecular geneticist assist the clinician? In this review we will show how careful study of the phenotype in the different subgroups can give important clinical insights into the subtypes of MODY. In addition we will try to outline how observations from the study of MODY may be significant for the understanding of type 2 diabetes in general.

The Definition of MODY

Before discussing phenotype/genotype relationships it is important to define MODY. If diabetes within a pedigree is likely to result from a mutation in a single gene the definition of MODY should include both early-onset non-insulin dependent diabetes mellitus (NIDDM) and autosomal dominant inheritance [1]. The criteria used in genetic studies of MODY families in the UK [2, 3] and France [4] were as follows:

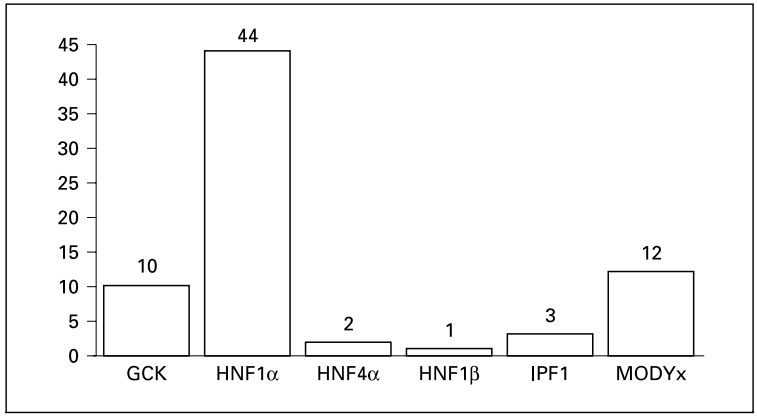

Fig. 1. The classification of 72 UK MODY families.

Early Onset Non-Insulin-Dependent Diabetes

Early onset was defined as at least one and ideally two members of the family being diagnosed before the age of 25 years although it was recognized that other family members may be diagnosed later. Patients were considered non-insulin dependent if 5 years after diagnosis they were not on insulin treatment or if they were treated with insulin they had significant circulating C-peptide.

Autosomal Dominant Inheritance

This is normally defined by three (and at least two) generations of diabetes with ideally cousins or second cousins showing the same phenotype of early-onset NIDDM. Families where both parents have NIDDM were normally excluded as this might represent an early-onset diabetes due to a 'double gene dose' from both parents [5].

The Relative Prevalence of the Different Subgroups of MODY

A national collection of MODY pedigrees has been in place in the UK since 1989. We have now been referred in excess of 200 families and genetic analysis of these families is ongoing. Figure 1 represents the relative contribution of the five genes in 60 UK pedigrees in whom mutations have been described: glucokinase (GCK), hepatocyte nuclear factor 1α (HNF-1α), hepatocyte nuclear factor 4α (HNF-4α), hepatocyte nuclear factor 1β (HNF-1β) and insulin promotor factor 1 (IPF-1). In addition there are a further 12

families whose genetic basis is at present undefined despite both sequencing and linkage analysis of the known MODY genes. We have designated these as MODYx.

Mutations in the hepatocyte nuclear factor 1α gene are the most common cause of MODY in the UK [6], followed by glucokinase gene mutations. The authors believe that for most of the different subgroups of MODY there is now a fairly clear phenotype. These are summarized in table 1.

Phenotype of Patients with Mutations in Glucokinase Genes

Since the initial observations in French [4] and English MODY families [2], over 40 mutations in the glucokinase gene have been described. These are distributed throughout the gene but tend to cluster in exons 5, 6, 7, and 8 which have been shown to code for the glucose binding site of the enzyme [7]. Clinical observations have given interesting insights into the function of the glucokinase gene in man.

Clinical Characteristics

Mutations of the glucokinase gene result in a discrete phenotype despite the wide variety of mutations [8]. These features are summarized in table 1. An elevated fasting blood glucose is a consistent feature and the majority of patients have blood glucose values within a tight range of 6–8 mmol/l (fig. 2). There is a mild deterioration of fasting blood glucose with age but blood glucose in patients in their 8th 9th decade still rarely exceeded 10 mmol/l [9]. This hyperglycaemia is present very early in life and recent observations showing altered fetal development would support these changes being present from birth. Patients with glucokinase mutations are usually asymptomatic throughout their life, and rarely have symptoms of hyperglycaemia. The vast majority will be detected by screening either for routine medicals, during pregnancy or family screening when MODY is suspected [2, 10]. In most patients the age of diagnosis is the age at which they are first tested. Whilst being diagnosed young is supportive of a glucokinase mutation the fact that a patient was only diagnosed in old age should not be used to exclude the diagnosis. In glucokinase, more than in any other type of diabetes, the age of diagnosis may be decades after the age of onset.

Treatment

Patients with glucokinase mutations rarely need any specific treatment [11]. The majority (>85%) are managed on diet alone. Avoiding unrefined carbohydrate may not be as important in NIDDM as most glucokinase patients

Table 1. Comparison of the different sub-types of maturity-onset diabetes of the young (MODY)

	HNF-4α (MODY1)	Glucokinase (MODY2)	HNF-1α (MODY3)	IPF-1 (MODY4)	HNF-1β (MODY5)	MODYx
Chromosomal location	20q	7p	12q	13q	17q	Unknown
Frequency in a large UK series	3%	14%	61%	4%	1%	17%
Mutations	No evidence of a common mutation	No evidence of a common mutation	C insertion in the exon 4 C tract (codon 291) in ≅25% of families with mutations	D76N found in many populations	No evidence of a common mutation	Not known
Penetrance of mutations at age 40 years	>80%	45% (>90% fpg>6 mmol/l)	>95%	>80%	?>95%	Not known
Onset of hyperglycaemia	Adolescence Early adulthood	Early childhood (from birth)	Adolescence Early adulthood	?Early adulthood	?Similar to HNF-1α	Uncertain
Severity of hyperglycaemia	Progressive may become severe	Mild with minor deterioration with age	Progressive may be severe	Limited data ?less penetrant than HNF-1α	May be severe	Variable
Micro-vascular complications	Frequent	Rare	Frequent	Little data	Frequent	Variable
Pathophysiology	β-cell dysfunction	β-cell dysfunction	β-cell dysfunction	?β-cell dysfunction	?β-cell dysfunction	β-cell dysfunction
Abnormality of glucose sensing?	No	Yes	No	?No	?No	Not known
Priming with hyperglycaemia	No	Yes	Yes – if normoglycaemic	Not known	Not known	Not known
Non-diabetes related features		Reduced birth weight	Low renal threshold and aminoaciduria	Pancreatic agenesis in homozygotes	Renal cysts Proteinuria Renal failure	

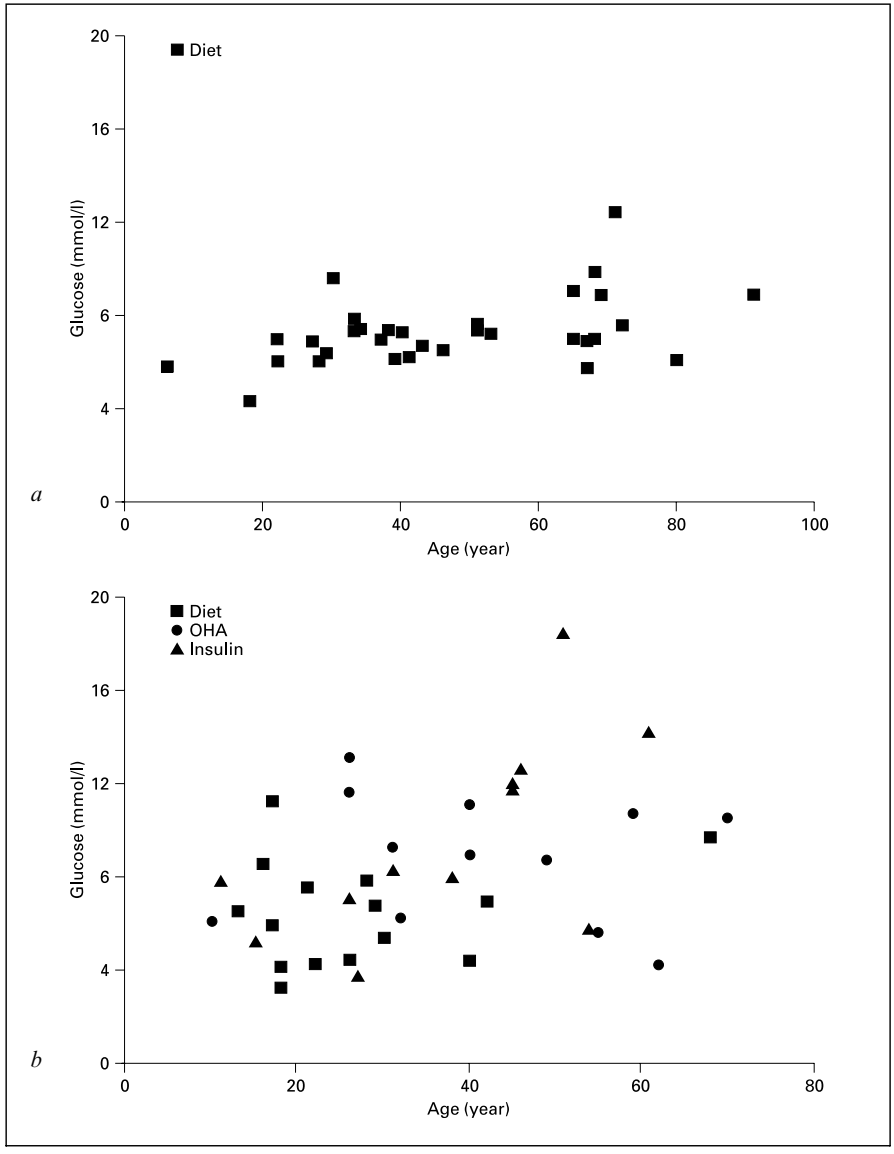

Fig. 2. The variation of blood glucose with age in patients with glucokinase mutations (*a*) and patients with HNF1α mutations (*b*).

on receiving 75g of glucose in an oral glucose tolerance test show little increment in their blood glucose [12]. Another interesting observation is that body mass index (BMI) makes little difference to the level of glycaemia in patients with glucokinase mutations [9]. This is in marked contrast to patients with NIDDM and HNF-1α mutations where weight loss can very effectively reduce glycaemia. In patients with glucokinase mutations this is probably because the fasting hyperglycaemia is due to a defect in glucose sensing and so, as in the normal subject, increased insulin resistance as a result of obesity results in compensatory hyperinsulinaemia rather than hyperglycaemia. The only time that glucokinase patients are likely to receive treatment is in pregnancy. In our series over 80% of patients received insulin during pregnancy. Our observations on fetal development suggest that this treatment was probably unnecessary when the fetus inherited the mutation, since fetal growth was similar to when neither mother nor fetus had a glucokinase mutation [13].

Complications

Patients with glucokinase mutations rarely suffer from microvascular complications [9, 14] probably as a result of their mild hyperglycaemia. The rare cases of proliferative retinopathy and diabetic retinopathy reported in the French series may represent coincidental conventional NIDDM in patients who also have a glucokinase mutation. It is hard to assess whether they have significant macrovascular complications as only approximately 500 patients have been described in the literature. In the Whitehall study an increased risk of macrovascular disease was seen in patients with impaired glucose tolerance so an increase might be expected in glucokinase subjects [15]. However, in contrast to impaired glucose tolerance (IGT), patients with glucokinase mutations have normal fasting lipids [9], are not significantly insulin resistant [2] and have less post-prandial hyperglycaemia [12].

Pathophysiology

In keeping with our understanding of the biochemistry of glucokinase, patients with glucokinase mutations have been shown to have β-cell dysfunction characterized as a defect in glucose sensing [2, 9, 16, 17]. First phase insulin response to an intravenous glucose bolus is well preserved [16]. First phase response to an oral glucose bolus is also well preserved explaining why a low glucose increment is seen in the oral glucose tolerance test (OGTT) [12]. Interestingly the abnormality in glucose sensing does result in a reduced first phase insulin response when a slow glucose infusion is given rather than a bolus [18].

Patients with glucokinase mutations do not show the features of insulin resistance seen in NIDDM. Insulin concentrations and plasma lipids are nor-

mal and in most series there is no increase in insulin resistance [2, 9]. In a large series of French patients using HOMA analysis Velho showed a small, but statistically significant, increase in insulin resistance in glucokinase patients with diabetes compared to those with fasting hyperglycaemia and to normal controls [19]. There were many possible explanations of this result: severe glucokinase mutations affected both β-cell functions and insulin sensitivity, the increased insulin resistance was secondary to glucose toxicity or these patients had an increase in the other genes that predispose to NIDDM resulting in their reduced glucose tolerance and their insulin resistance. One area which is clear is that patients with glucokinase mutations have reduced hepatic glycogen synthesis showing that glucokinase is a rate determining step in the liver as well as in the pancreatic β-cell [20].

Classification

The same glucokinase mutation has been identified in patients who were defined as having either MODY, NIDDM or gestational diabetes [21, 22] depending on when the diagnosis was made. There was no evidence of a different phenotype in these three groups [23]. This shows the difficulties of a clinical classification where the time of diagnosis determines the classification of the type of diabetes. Patients with a glucokinase mutation appeared to have a discrete phenotype and hence a classification based on aetiology had been proposed [23, 24]. The American Diabetes Association has recently supported such an aetiological classification proposing 'Genetic defects of beta-cell function; Chromosome 7, glucokinase' [25].

Prevalence

The prevalence of glucokinase mutations is difficult to assess as the mild hyperglycaemia and absence of symptoms means patients are frequently not diagnosed. Large-scale population studies to assess the prevalence have not been done. In the white Caucasian population in the UK, approximately 2% of the population will be diagnosed as having gestational diabetes and, of these, approximately 2–5% [22, 26] will have glucokinase mutations. This would suggest a prevalence of 0.1–0.04%.

The prevalence of MODY patients with glucokinase mutations varies between 56% in France [11], to 12.5% in the UK (Beards, Ellard and Hattersley, unpublished observation) and $<1\%$ in Japan [27]. This may be the result of genetic differences between races but also there were important differences in how the families were ascertained. The high prevalence in French MODY probably reflects that glucose tolerance testing was performed in asymptomatic young relatives in NIDDM families recruited by media appeal direct to patients [28]. The UK and Japanese studies were predominantly of symptomatic pa-

tients attending hospital outpatients and so patients with glucokinase mutations are less prevalent. These patients are most likely to come to medical attention when pregnant as this is a time when asymptomatic individuals are routinely screened. If patients with gestational diabetes are selected on specific clinical criteria the prevalence may be high. In UK patients with a past history of gestational diabetes who also had persistent fasting hyperglycaemia, an increment on an OGTT <3 mmol/l and a family history of mild hyperglycaemia, the chance of finding a mutation is approximately 65% (Beards, Ellard and Hattersley, unpublished observation).

Phenotype of HNF-1α Mutations (MODY3)

Bell's group in Chicago and collaborators from France, UK, Japan, Denmark and USA showed that the transcription factor HNF-1α was the MODY3 gene in December 1996 [29]. Mutation detection in UK [6], German [30], French [31], Danish [32], Italian [33], Finnish [34], North American [34] and Japanese [35] MODY pedigrees confirmed that HNF-1α mutations are the commonest cause of MODY. In these series approximately 25% of the families with an HNF-1α mutation had a C insertion in the C tract in exon 4 (mutation p291fsinsC). This common mutation was thought to result from a hot-spot for spontaneous mutations rather than representing a single distantly related family [30, 34, 36]. In the UK the C insertion accounted for 9 out of 60 MODY subjects defined on strict criteria and only one out of an additional 42 subjects defined by less stringent criteria [36]. This suggests a frequency in UK MODY of between 10 and 15%. Over 60 other mutations have been found scattered throughout the gene and consist of frameshift, missense, nonsense and splice site mutations.

Clinical Characteristics

The phenotype associated with HNF-1α mutations is markedly different from the glucokinase phenotype (see table 1). Most subjects under the age of 10 years have a normal fasting blood glucose and normal glucose tolerance. In adolescence and early adulthood, subjects with HNF-1α mutations may show no, or only minimal elevation, of their fasting blood glucose but be diabetic on the 2 hour value in an OGTT. The clinical diagnosis of diabetes is at a mean age of 23 years. Most subjects present with osmotic symptoms [10]. This may be as a result of the reduced renal threshold seen in these patients [37].

Penetrance of Mutations

HNF-1α mutations have a high penetrance with the majority of subjects having diabetes by the age of 25 years [6]. However some subjects found to

carry HNF-1α mutations were not diagnosed until middle or old age and it is difficult to determine whether this is due to a delayed diagnosis, a later onset of diabetes or both. The subjects with HNF-1α mutations who do not have diabetes on testing are frequently young and are likely to develop diabetes later in life [6]. There are some individuals who have reached middle age without having diabetes and these unusual cases probably represent true non-penetrance of the gene [38]. These subjects have a low BMI and so can compensate for their β-cell defect by being sensitive to the insulin they do produce.

Treatment

Once patients with HNF-1α mutations develop diabetes they show progressive deterioration in their glucose tolerance in contrast to the relatively stable mild hyperglycaemia seen in patients with glucokinase mutations (fig. 2). They are also likely to have increasing treatment requirements as they get older. In a group of 142 UK diabetic subjects with HNF-1α mutations approximately one third are treated by diet, one third treated with oral agents and one third treated with insulin. The patients on insulin tend to be older (see fig. 2) and more obese than those on diet and oral agents. Some patients are misdiagnosed as having insulin-dependent diabetes because they present with osmotic symptoms in their late teens or early twenties with random blood glucose in the region of 15–20 mmol/l. In this situation some have been put straight on to insulin treatment. Such patients may be recognized by their autosomal dominant family history, their excellent glycaemic control despite relatively low doses of insulin (usually less than 0.5 units per kg) and an absence of ketoacidosis even when insulin is omitted.

Complications

Microvascular complications can develop, particularly retinopathy [39]. In the UK series 20% of subjects with HNF-1α mutations had significant retinopathy as shown either by loss of sight or laser therapy. This means that it is important to strive for excellent glycaemic control. The pattern of complications and also vascular dysfunction is similar to that seen in type 1 diabetes [40]. In both these sub-groups of diabetes the primary pathophysiology is in the β-cell with insulin resistance and dyslipidaemia are frequently not seen.

Pathophysiology

They pathophysiology in patients with HNF-1α mutations is a progressive β-cell defect. Physiological experiments have shown that the patients who have inherited the HNF-1α mutation but are not yet diabetic, show adequate insulin

when fasting but are unable to increase their insulin secretion as glucose levels rise [41]. Prolonged hyperglycaemia (18 hours) primes the β-cell in these non-diabetic individuals. Even in the presence of mild hyperglycaemia the priming of the β-cell is lost. Unlike glucokinase there is no defect in the sensing of glucose [17].

Classification

Classification of HNF-1α subjects is much easier than glucokinase patients as the majority present with symptomatic diabetes in adolescence or early adulthood. The recent ADA classification now suggests an aetiological diagnosis should be used e.g. genetic defect in β-cell function; chromosome 12 HNF-1α.

Prevalence

Mutations in HNF-1α are the commonest cause of diabetes in European Caucasian patients with MODY. If strict criteria are used then a prevalence of 50–90% is seen, but the prevalence is lower when less stringent criteria are used. We estimate that these patients account for about 1–2% of a Caucasian diabetic clinic although many may not be formally recognized as MODY [42]. This would result in a population frequency of approximately 0.02–0.04%.

Phenotype of HNF-4α Mutations (MODY1)

HNF-4α mutations are relatively rare with only a small number of families in the literature. By far the best characterized family is the RW pedigree studied by Fajans over many years. The in-depth study of this family has given many crucial insights which are reviewed in the accompanying article from Fajans.

Most of the features seen in this pedigree seem to be generalizable to other families with HNF-4α mutations. In French and English families with HNF-4α mutations a latter age of onset has been described than seen in patients with HNF-1α mutations [43, 44]. This suggests a reduced age-related penetrance at least in these families. This may be a feature of the specific mutations seen or alternatively a result of the polygenic and environmental background on which the mutations occurred.

Phenotype of Mutations in the Insulin Promoter Factor 1 (MODY4)

Elegant clinical science led to the identification of the insulin promoter factor 1 (IPF-1) also known as PDX-1 gene as a cause of MODY. The key

role of IPF-1 in the development of the pancreas was shown by the pancreatic agenesis found in transgenic mice that were homozygous for a knockout of IPF-1. Stoffers and colleagues [45] studied a child with pancreatic agenesis and found a homozygous frameshift mutation. It was noticed that there were multiple generations of diabetes on both sides of the family and they went on to show that the heterozygous frameshift mutation co-segregated with NIDDM in both parents' families, with a total LOD score of 3.43 [46].

Clinical Features

Defining the clinical phenotype is difficult as there is only one published family to date. Whilst this family did meet standard criteria for MODY with one of the eight diabetic members diagnosed under the age of 25, there was evidence that this gene was less penetrant than a typical HNF-1α mutation. Two individuals had inherited the mutation but apparently had normal glucose at 22 and 23 years and the mean age at diagnosis was 35 years (range 17–68) which is considerably higher than the mean age at diagnosis of HNF-1α mutations of 23 years. At present there is no evidence regarding the clinical characteristics or underlying pathophysiology in this family.

Prevalence

In France [47] and Japan [48] no IPF-1 mutations were found in maturity-onset diabetes of the young probands. In the UK a single missense mutation in IPF-1 was found in one of twelve individuals who were known not to have a mutation in the major MODY genes but there was not clear co-segregation with diabetes (Macfarland, Ellard, Docherty and Hattersley, unpublished data). In the UK three out of 36 individuals (8%) with NIDDM diagnosed between 25 and 45 years had missense mutations in IPF-1. These mutations were shown to result in reduced activation of the insulin gene in response to elevated glucose concentrations in vitro, suggesting they were likely to be pathogenic diabetes mutations (Macfarland, Ellard, Docherty and Hattersley, unpublished data).

Pathophysiology

Glucose tolerance tests in apparently unaffected individuals with IPF-1 mutations who had a normal fasting blood glucose and HBA1c levels showed higher 2-h glucose values than matched controls. Insulin secretion, particularly first phase insulin secretion, was reduced. This data suggests that mutations in insulin promotor factor 1 result in reduced β-cell function. In general the effect is less profound than that seen in HNF-1α mutations and young adults who are not markedly insulin resistant may not be diabetic despite having reduced insulin secretion in response to a glucose load. It is likely that variation

of the severity of the mutation explains, at least to some extent, the variation in phenotype. It seems likely that IPF-1 mutations are more common in early-onset NIDDM rather than strict MODY. In these early-onset subjects IPF-1 mutations may well be a part of a polygenic predisposition to NIDDM rather than a simple monogenic aetiology seen in MODY.

Phenotype of HNF-1β Mutations (MODY5)

As HNF-1β acts as a hetero-dimer with HNF-1α it was a clear candidate gene for MODY. The first description in a subject with MODY was in a Japanese family [49]. The fact that over 60 subjects have been screened to find this one individual was an indication that such mutations were relatively rare and certainly considerably less frequent than HNF-1α mutations. No mutations in HNF-1β were found in strictly defined MODY in the UK population [50]. Despite the low prevalence the study of the phenotype in these families has given some fascinating new insights into our understanding of the role of transcription factors.

The Diabetic Phenotype

Patients with HNF-1β mutations appear to present with early-onset with a similar severity to patients with mutations in the HNF-1α gene [49]. The low numbers of families described makes it difficult to detect any subtle differences. The fact that some subjects have proliferative retinopathy is an indication that these patients can be sufficiently hyperglycaemic to develop marked microvascular complications. Detailed physiological studies have not been reported on these patients but it seems very likely that there is a β-cell defect rather than a predominant abnormality in insulin resistance.

The Renal Phenotype

The most fascinating observation in HNF-1β families has been the co-existent renal problems which co-segregate with the HNF-1β mutation. In the initial description it was noted that patients with mutations had end-stage renal failure and proteinurea [49]. A subsequent publication has shown that these features were not merely a consequence of diabetic nephropathy but are a phenotype in their own right. In the Japanese family it was shown that all members of a family who had an HNF-1β mutation had at least one renal manifestation [51]. These included end-stage renal failure, cysts in the kidney on ultrasound and proteinurea.

These renal manifestations were seen in young children with mutations at a time when they were not diabetic [51]. Preliminary observations in a UK

family showed that this defect is present as early as 17 weeks of pregnancy and histological examination suggests that the underlying defect is failure of nephron development (Hattersley, Ellard, Allen, Frayling, unpublished observation). Within our family there was a great variation of the renal phenotype in three affected members suggesting that other environmental and polygenic influences alter the phenotype of the kidney defect.

Phenotype of MODYx Families

There is still a significant proportion of MODY families where the genes have not been defined. In the UK and France this accounts for about 15–20% of MODY but it is considerably higher in Japan. One of the original pedigrees described by Tattersall, pedigree M [1], has been shown not to be linked to any of the five known MODY loci.

In the absence of an underlying genetic defect it is difficult to make precise statements on the phenotype. Some families who do not have a mutation in the known MODY genes have been defined by less rigorous criteria. In such families diabetes may result from a high concentration of polygenic influences rather than a single gene. In those families where there is likely to be a single gene mutation segregating there still appears to be heterogeneity. In the French series the phenotype of their MODYx families was intermediate between the glucokinase and HNF-1α phenotype while the UK MODYx families have a severe phenotype similar to the patients with HNF-1α gene mutations. In all the UK families there is a β-cell defect. Genetic analysis using a combination of reverse genetics and candidate gene studies will help to define these families further. Key candidate genes are likely to be those involved in gene transcription, pancreatic development or glucose sensing in the pancreatic islet.

Insights from the Effect of Mutations in the Glucokinase Gene on Fetal Growth

Observations in MODY subjects can give broad insights into the function of the causative gene. Fetal insulin secretion is one of the key determinants of fetal growth acting mainly during the third trimester when the weight of the fetus increases markedly. The clearest clinical example of this is the macrosomic children born to mothers with diabetes in pregnancy. Pederson proposed that this macrosomia did not result from a direct increase in the transfer of nutrients, but was mediated indirectly by increased fetal

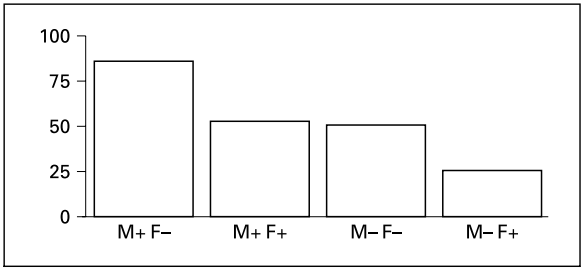

Fig. 3. The centile birth weight of children in families where one parent has the glucokinase mutation. The results are classified by whether the fetus has the mutation (F+/F–) and whether the mother has the mutation (M+/M–). Data taken from reference 13.

insulin secretion in response to fetal sensing of maternal hyperglycaemia [52].

Fetal insulin related growth not only reflects maternal glycaemia but also fetal genetic factors which regulate the fetus's insulin secretion. As the fetus produces insulin in response to the maternal glucose level, we hypothesized that a defect in sensing of the maternal glucose by the fetal pancreas as a result of mutations in the glucokinase would result in reduced fetal growth.

Observations of fetal development in families with glucokinase mutations confirmed this hypothesis [13]. At birth a fetus who inherited a glucokinase mutation was lighter than his sibling who did not in 90.4% of sibling pairs and the mean difference was 521g. There was interaction between fetal and maternal effects upon fetal growth. Maternal hyperglycaemia, due to a mother having a glucokinase mutation, resulted in the fetus being 601g heavier. Figure 3 shows the effects of fetal and maternal mutations on mean birth weight centiles. When both mother and fetus had the glucokinase mutation the two opposing effects cancelled out and the baby was of normal weight [13].

These observations are important because they establish that glucokinase plays a key role in the sensing of glucose by the fetus as well as after delivery. Preliminary data suggests that the effect of the glucokinase mutation is seen in the third trimester. This result has also lead to a more wide-ranging hypothesis which we describe as the 'fetal insulin hypothesis'. This hypothesizes that genetic defects that alter either fetal insulin secretion or insulin action could, by reducing insulin-mediated growth, reduce birth weight. This hypothesis is a possible explanation of the association seen between low birth weight and diabetes in adult life [53].

Using Molecular Genetic Information in the Management of Patients with MODY

We have now reached a level of scientific understanding such that molecular genetic information can help in the clinical management of patients. For patients with diabetes, a molecular genetic diagnosis will allow a definite diagnosis of MODY to be made, the sub-type to be classified and hence help with prediction of the likely prognosis and clinical course. A young child with slightly raised fasting glucose can be predicted to have a very different clinical course if they have a glucokinase mutation, mutation in HNF-1α or neither of these and a high level of islet cell antibodies. An area of uncertainty is the degree of medical supervision necessary for patients with glucokinase gene mutations. Certainly close follow up of children who have only mildly elevated fasting glucose levels is not warranted and repeated contact with a diabetic clinic predominantly dealing with IDDM may be detrimental. We cannot assume that older patients will not have diabetic complications, particularly as there is no evidence that these patients are protected from developing polygenic NIDDM. Annual follow up of adults with measurement of HBA1c annually is probably all that is required. Only the few patients with an elevated HBA1c will require more careful follow up.

Mutation-based genetic testing can predict, with a high degree of accuracy, whether an unaffected first degree relative is likely to develop MODY in the future. Full genetic counselling should be provided for individuals who seek predictive genetic testing. The child of a MODY patient may find it helpful to know whether or not they have inherited a mutation especially if they reach the age of 25 and are apparently unaffected. If they do not have the mutation then the risk of diabetes would be the same as that of the population and hence blood screening on an annual basis would no longer be necessary. Testing of young children may also be beneficial but this is considerably more controversial. This area of clinical practice needs to be studied carefully before its widespread introduction into clinical use.

Conclusion

The MODY gene story has developed from the early clinical description. Mutations in five discrete genes have been shown to cause MODY: glucokinase, hepatocyte nuclear factor 1α (HNF-1α), hepatocyte nuclear factor 4α (HNF-4α), hepatocyte nuclear factor 1β (HNF-1β) and insulin promoter factor 1 (IPF-1). The description of these genes has allowed us to understand much of the clinical and physiological heterogeneity seen in MODY. Molecular

genetic testing allows new possibilities for the classification of MODY and also possibly prediction and eventually prevention of diabetes within these families.

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Andrew T. Hattersley, MD, Department of Vascular Medicine (Diabetes Research),
Postgraduate Medical School, Barrack Road, Exeter, EX2 5AX (UK)
Tel. +44 1392 403082, Fax +44 1392 403027, E-Mail a.t.hattersley@exeter.ac.uk

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Medical Genetics of MODYs

Philippe Froguel [a], *Gilberto Velho* [b]

[a] Institute of Biology-CNRS EP10, Institut Pasteur de Lille, et
[b] INSERM U-342, Hôpital Saint Vincent de Paul, Paris, France

Introduction

Maturity onset diabetes of the young (MODY) is a genetically and clinically heterogeneous subtype of noninsulin-dependent diabetes mellitus (NIDDM) characterized by early onset, autosomal dominant inheritance and a primary defect in insulin secretion. To date, five MODY genes have been identified on chromosomes 20q, 7p and 12q, 13q12.1 and 17cen-q21.3 designated MODY1/Hepatocyte Nuclear Factor 4 alpha (HNF-4α, MODY2/glucokinase (GCK), MODY3/Hepatocyte Nuclear Factor 1 alpha (HNF-1α, MODY4/Insulin Promoter Factor 1 (IPF-1) and MODY5/Hepatocyte Nuclear Factor 1 beta (HNF-1β). Moreover there are likely to be additional MODY genes to identify. Mutations in glucokinase/MODY2 result in mild chronic hyperglycemia due to reduced pancreatic β cell responsiveness to glucose, and decreased net accumulation of hepatic glycogen and increased hepatic gluconeogenesis following meals. In contrast, MODY1 and MODY3 are characterized by severe insulin secretory defects, and by major hyperglycemia associated with microvascular complications. The identification of the role of mutations in transcription factors in MODY now opens entirely new perspectives in the understanding of the molecular mechanisms of glucose homeostasis and of its disorders.

MODY Is an Autosomal Dominant Form of Type 2 Diabetes

Type 2 diabetes is a genetically, metabolically and clinically heterogeneous syndrome having multifactorial etiologies [1]. Although in most cases type 2 diabetes seems to be a polygenic disorder, genetic approaches have recognized monofactorial forms of type 2 diabetes [2–5]. Among those highly genetically

inherited forms of type 2 diabetes, maturity onset diabetes of the young (MODY) was the most intensively investigated in the last few years [6]. MODY is characterized by familial type 2 diabetes with an early age of onset (childhood, adolescence or young adulthood) and autosomal dominant inheritance, associated with insulin-secretion defects. The well-defined mode of inheritance with high penetrance and the early age of onset of diabetes which allows the collection of multigenerational pedigrees made MODY an attractive model for genetic studies of type 2 diabetes. The variable phenotype of subjects with MODY suggested that the disorder was genetically heterogeneous, an observation that was confirmed by genetic studies. Mutations in genes on chromosomes 20q, 7p and 12q, 13q12.1 and 17cen-q21.3 designated MODY1/Hepatocyte Nuclear Factor 4 alpha (HNF-4α [7, 8], MODY2/glucokinase (GCK) [9, 5, 10], MODY3/Hepatocyte Nuclear Factor 1 alpha (HNF-1α) [11, 12], MODY4/Insulin Promoter Factor 1 (IPF-1) [13, 14] and MODY5/Hepatocyte Nuclear Factor 1 beta (HNF-1β) [15] cause most of the MODY cases. Moreover there are likely to be additional MODY genes since there are families in which MODY does not cosegregate with markers tightly linked to the 5 known MODY loci [11, 16].

The prevalence of MODY is still unknown, but it seems to have a worldwide distribution [17, 10]. Recent studies suggest that 2–5% of patients with type 2 diabetes may in fact have MODY [15]. Moreover, MODY was found in approximately 10% of the Caucasian type 2 diabetes families we have collected in France [5]. The relative prevalences of the different MODY subtypes remain uncertain. Analyses of a set of 67 MODY families that we have now collected in France show that 63% (42 families) have the MODY2 and 21% (14 families) have the MODY3 subtypes [16, 10, 13]. Thus, the additional unknown MODY locus or loci represent 16% of the families from our panel. In contrast with our results, Frayling and coworkers have observed that MODY2/glucokinase represent only 11% of cases of MODY in a panel of British kindreds, while HNF-1α mutations are highly prevalent (73%) in that population [17]. These contrasting results may be due to differences in the genetic background of the two populations, or else, may reflect, at least partly, ascertainment bias in the recruitment of families.

Glucokinase and the Glucose Sensing Pathway

Glucokinase phosphorylates glucose to glucose-6-phosphate in pancreatic β-cells and hepatocytes, and plays a major role in the regulation and integration of glucose metabolism [18]. More than 80 different GCK mutations have been observed to date [10, 19]. Expression studies have shown that the enzymatic

activity of the mutant proteins was impaired, with either a decrease of V_{max} and/or a decrease of the affinity of the enzyme for glucose [20]. Impairment in the enzymatic activity of mutant GCK results in decreased glycolytic flux in pancreatic β-cells [21]. This defect translates in vivo as a glucose sensing defect leading to an increase in the blood glucose threshold that triggers insulin secretion [22], and a right shift in the dose response curve of glucose-induced insulin secretion [23]. Comparison of insulin secretion rates (ISR) at different glucose levels demonstrated that glucokinase-deficient subjects present an average 60% reduction in insulin secretion for a given glucose level. Interestingly, subjects carrying mutations that affect only mildly the enzymatic activity of glucokinase in vitro present a much lower reduction in ISR as compared to controls than subjects carrying severe mutations. The release of insulin in response to arginine is usually well preserved [24], which suggests that this secretory defect is indeed related to glucose sensing.

Decreased net accumulation of hepatic glycogen and augmented hepatic gluconeogenesis following meals were observed in glucokinase-deficient subjects [25]. Moreover, glucokinase-deficient subjects have abnormal suppression of hepatic glucose production by physiological levels of insulin during an euglycemic clamp [28] and they present a decreased hepatic glucose cycling, and an endogenous glucose production that is abnormally high in relation to their plasma glucose levels with a blunted suppression after oral glucose administration [29]. In this regard, attenuation of pancreatic and hepatic glucokinase expression in transgenic mice results in pancreatic and hepatic defects comparable to those observed in GCK-deficient subjects [30–32].

Despite these multiple defects in the pancreas and the liver, the hyperglycemia associated with GCK mutations is often mild, with fewer than 50% of subjects presenting overt diabetes [10]. However, it develops during the early years of life (youngest age of diagnosis: 12 months) and its penetrance in the affected families is very rapidly complete in that the individuals who carry the mutation are nearly always affected before puberty [10]. There is a lower prevalence of proliferative retinopathy, proteinuria, and peripheral neuropathy in MODY2 than in other subtypes of MODY and late-onset type 2 diabetes [33, 10]. This may be a consequence of the relatively small increase in blood glucose levels and of the low prevalence of hypertension in these subjects.

Mutations in Transcription Factor Genes Cause MODY

Positional cloning of MODY genes have led to the identification of mutations in four transcription factors, so far: HNF-1α and HNF-1β, which functions as a homodimer or a heterodimer with HNF-1α, HNF-4α, and IPF-1

[13, 14]. The hepatocyte nuclear factors (HNFs), nuclear proteins, initially found to be expressed in the liver, are well known to modulate the expression of many target genes (including albumin, apo C3...) in this organ, but their role in pancreatic islets and in the kidney was until recently completly ignored [34]. In this regard, the HNFs were not candidate genes for diabetes, and their role in diabetes was only found through pure genetic approaches. However, transcription factors gene targeting in animals have recently demonstrated that many of these islet-expressed genes have a key role in fetal development, like HNF-4α, and in β-cell differentiation, proliferation and neogenesis, like IPF-1. These islet-expressed nuclear factors should be considered major candidate genes for diabetes in human.

Most of the nuclear factor mutations associated with MODY were identified in HNF-1α: more than 80 different mutations in HNF-1α coding region or in its promoter were found in various populations where they cosegregated with diabetes in MODY families or in atypical (non-autoimmune) forms of type 1 diabetes [12, 13, 16, 17, 35–38 and personal unpublished data]. An insulin secretory defect in the absence of insulin resistance was observed in diabetic and nondiabetic carriers of MODY3 mutations [39, 40], suggesting that HNF-1α is indeed implicated in pancreatic β-cell function. However, the mechanisms and the target genes associated with this β-cell defect remain mostly unknown. In contrast with the heterozygous mice lacking HNF-1α which has a normal phenotype, MODY3 subjects carry only one copy of the HNF-1α mutation, suggesting that they have a dominant negative effect. However, it seems that only mutations in the transactivation domain have a dominant negative effect on HNF-1α transactivation potential, mutations located elsewhere in the protein only suppressing or even decreasing HNF-1α activity (Martine Vaxillaire, unpublished data).

The clinical phenotype of MODY3 resembles late age of onset type 2 diabetes in its natural history [33], with subjects rapidly progressing from impaired glucose tolerance to overt diabetes, and with deterioration of insulin secretion. In contrast to the usually mild hyperglycemia due to glucokinase deficiency, MODY3 is a severe form of diabetes, often evolving to insulin requirement. Proliferative retinopathy was observed as frequently in MODY3 as in late age of onset type 2 diabetes subjects. A trend towards a higher prevalence of proteinuria in MODY3 subjects was also observed. However, unlike type 2 diabetes with late age of onset, the MODY3 subtype is associated with a low prevalence of obesity, dyslipidemia, and arterial hypertension. Moreover, unlike MODY2, the MODY3 is not a disease of childhood, with hyperglycemia usually developing after puberty.

In contrast to MODY3/HNF-1α, MODY1/HNF-4α seems to be very unfrequent. The American RW pedigree, consisting in 360 identified members,

including 72 known affected subjects, was the first family found to carry a mutation in the nuclear receptor HNF-4α [8], which is a member of the steroid/thyroid hormone receptor superfamily and upstream regulator of HNF-1α expression. The affected individuals from the RW pedigree also present a severe form of diabetes which requires insulin therapy in about 30% of cases and is associated with microvascular complications [41]. A primary pancreatic β-cell defect was observed in these subjects [42, 43]. Additional mutations in the HNF-4α gene was identified in few British, Danish, and Finnish pedigrees with MODY [44 and unpublished data from Leif Groop and Oluf Pedersen]. No mutation in MODY1/HNF-4α were identified in France, to date.

Mutations in MODY5/HNF-1β were recently described in 3 families from Japan, Norway and UK [15, and unpublished data from Graeme Bell and Andrew Hattersley]. In these pedigrees HNF-1β mutations were associated with diabetes and severe kidney disease which may appear before glucose intolerance. Polycystic renal disease and/or particular histological abnormalities showing meganephrons were present in some subjects, suggesting that this gene could play a major role in kidney development and nephron differentiation.

All of these transcription factor genetic defects lead to abnormalities of glucose homeostasis, and thereby promote the development of chronic hyperglycemia, through alterations in insulin secretion and possibly, in the development of the pancreatic islets. In this regard, a deletion mutation in the homeodomain transcription factor IPF-1, which is also known as IDX-1, STF-1 and PDX-1, protein was found to cosegregate with MODY in a large kindred presenting a consanguineous link [14]. The average age of onset of chronic hyperglycemia in this kindred (35 years) is higher than in typical MODY pedigrees, but it ranges from 17 to 67 years. This IPF-1 Pro63fsdelC mutation results in a premature stop codon and a protein lacking a domain which is crucial for DNA binding. In this pedigree the Pro63fsdelC mutation when present in a homozygous state is responsible for pancreatic agenesis [13]. Indeed, the homeodomain-containing protein IPF-1 is critically required for the embryonic development of the pancreatic islets as well as for transcriptional regulation of endocrine pancreatic tissue-specific genes in adults, such as the insulin, glucose transporter-2 (GLUT2) and glucokinase genes in β-cells, and the somatostatin gene in d-cells. IPF-1 is normally expressed in all cells of the pancreatic bud, and its absence in mice arrests development at the bud stage leading to pancreatic agenesis [45].

The role of transcription factors in the development of the more common forms of late-onset type 2 diabetes is still under investigation. Linkage analyses studies and screening for mutations have excluded a role as major susceptibility genes for these 4 MODY genes. However, mutations in HNF-1α were identified

in Black Americans, Japanese and Caucasians [46 and Oluf Pedersen and Philippe Froguel, unpublished data] with atypical forms of acute-onset non-autoimmune diabetes. A common polymorphism in HNF-1α may be associated with mild insulin secretion defects [47]. A single mutation in HNF-4α [48] and several mutations in IPF1 were recently identified in French type 2 diabetic families with typical late-onset NIDDM (El Habib Hani, unpublished data). In some of these families, deleterious mutations significantly impairing the transactivational activity of these transcription factors were responsible for monogenic-like forms of type 2 diabetes with late age of onset, which may represent an intermediary phenotype between MODY and the most common forms of type 2 diabetes.

What Did We Learn from the Genetics of MODY?

MODY is a genetically heterogeneous subtype of type 2 diabetes characterized by early onset, autosomal dominant inheritance and a primary defect in insulin secretion. MODY is a relatively frequent disorder, as recent studies suggest that 2–5% of patients with type 2 diabetes may in fact have MODY. The nature or position of different mutations in MODY genes could partially explain diverse phenotypic presentations of diabetes in MODY pedigrees. Indeed, mutations in sites affecting in a major way the function of the gene product might be sufficient to induce glucose intolerance early in life, regardless the absence of other predisposing factors. On the other hand, mutations affecting this function only mildly, could segregate in kindreds without leading to hyperglycemia at an early age. Later in life, other genetic or environmental factors could contribute to the development of diabetes.

The identification of glucokinase as a diabetes susceptibility gene have provided a major impulse for the reassessment of the physiological role as a glucose sensor and the understanding of pathophysiological importance of this key enzyme of glucose homeostasis. More recently, the recognition of role of transcription factors in MODY opened new perspectives in the understanding and treatment of type 2 diabetes and of the mechanisms of glucose homeostasis. Only a small proportion of diabetic patients, mostly members of typical MODY families, have inherited mutations in known islet transcription factor genes which are sufficient to cause diabetes. However, one could speculate that an impaired expression of islets master nuclear factors like HNF-3β, possibly due to insulin resistance at the β-cell level, and/or a secondary impairment of their transactivational activity, due to chronic exposure to high concentrations of glucose or free fatty acids, may also contribute to the development of type 2 diabetes as well. Indeed, many studies have suggested that chronic

hyperglycemia and high FFA levels may contribute to islet glucotoxicity and lipotoxicity. It was recently shown that chronic exposure of insulin-secreting HIT-T15 cells to elevated glucose concentrations was associated with decreased activity of the HNF-1 response element (Vincent Poitout, unpublished data). These data might provide novel information on the mechanisms by which chronic hyperglycemia alters β-cell function in type 2 diabetes, not only by decreasing insulin gene transcription, but also by affecting a transcription factor potentially implicated in the regulation of insulin secretion in response to glucose.

The identification of MODY genes has obviously contributed to improve our knowledge of the glucose homeostasis, and may help to the definition of targets for new drugs for the treatment of diabetes. In this regard, MODY can be considered as a paradigm of type 2 diabetes.

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Dr. Philippe Froguel, Institute of Biology-CNRS EP10, Institut Pasteur de Lille,
1, rue Calmette, F–59000 Lille (France)
Tel. +33 3 20 87 79 54, Fax +33 3 20 87 72 29, E-Mail froguel@xenope.pasteur-lille.fr

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Coupling of SUR1 and $K_{IR}6.2$ Specify the Properties of β-Cell K_{ATP} Channels

Andrey P. Babenko[a], *Nidhi Sharma*[b], *Lydia Aguilar-Bryan*[b], *Gabriela Gonzalez*[a], *Joseph Bryan*[a]

Departments of Molecular and Cellular Biology[a] and Medicine[b] Baylor College of Medicine, Houston, Tex., USA

Membrane electrical activity plays a key role in the regulation of insulin secretion. ATP-sensitive potassium channels, K_{ATP} channels, couple changes in glucose metabolism to β-cell membrane potential. Increases in the ATP/ADP ratio, the result of increased glucose metabolism, close K_{ATP} channels, resulting in membrane depolarization and increased Ca^{2+} influx which triggers a cascade of events that culminate in insulin secretion. β-cell K_{ATP} channels are heteromultimers of $K_{IR}6.2$ and a sulfonylurea receptor, SUR1, an ATP-binding cassette (ABC) protein with several isoforms. $K_{IR}6.2$ forms a channel pore whose spontaneous activity and ATP sensitivity are modulated by the receptor. The inhibition of K_{ATP} channel activity by sulfonylureas has been widely employed as a treatment for noninsulin dependent diabetes mellitus (NIDDM), while the loss of channel activity as a result of mutations in SUR1 or $K_{IR}6.2$ causes a recessive form of persistent hyperinsulinemic hypoglycemia of infancy (PHHI), a neonatal disorder characterized by unregulated insulin release despite severe hypoglycemia. This paper reviews our recent studies directed at understanding the structural elements for the coupling between SUR1 and $K_{IR}6.2$, and how this coupling is involved in ATP- and sulfonylurea-inhibitory gating.

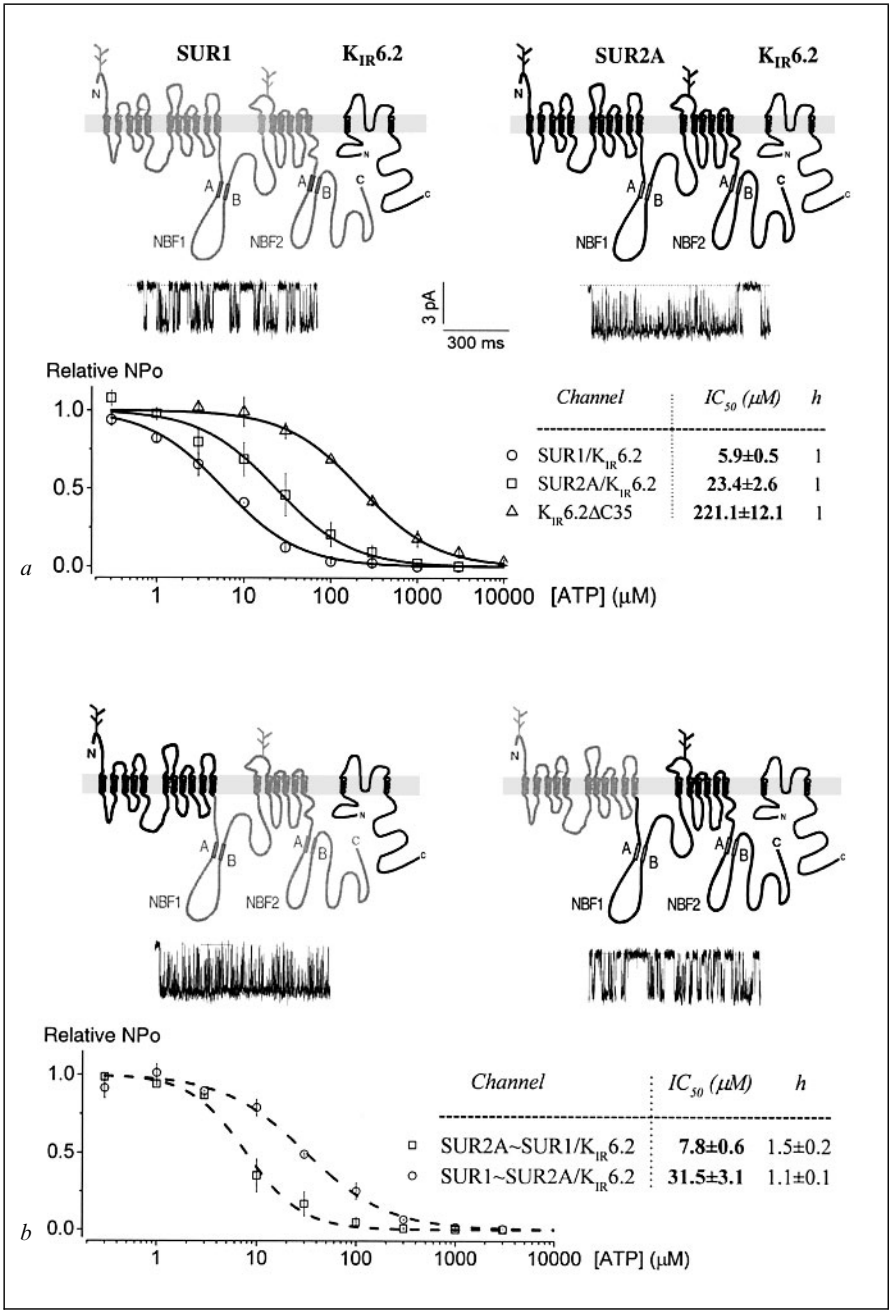
Introduction

Ionic mechanisms play a central role in the control of insulin secretion and the pharmacologic manipulation of the β-cell membrane potential is an

effective means of modulating insulin release. The 'classical' ionic mechanism for coupling increased glucose metabolism to membrane electrical activity holds that (a) glucose metabolism causes an increase in the ATP/ADP ratio, or $[ATP]_i$, causing ATP-sensitive K^+ channels (K_{ATP} channels) to close; (b) which leads to depolarization of β-cell membranes, activation of voltage-gated Ca^{2+} channels, Ca^{2+} influx, and a rise in the free cytosolic Ca^{2+} concentration, $[Ca^{2+}]_i$; and (c) this increase in calcium stimulates the exocytosis of insulin (see [1] for review). Although there is considerable evidence (see for example [2–4]) for nonionic mechanisms which contribute to the control of insulin secretion, recent work has shown that loss of K_{ATP} channel activity causes the recessive form of persistent hyperinsulinemic hypoglycemia of infancy, a disorder of newborns characterized by inappropriately high insulin levels despite persisting hypoglycemia indicating these channels are essential (for reviews see [5–9]), The cloning of K_{ATP} channel subunits opened the way for a molecular analysis of these channels. The current picture indicates β-cell K_{ATP} channels are octamers, $(SUR1/K_{IR}6.2)_4$, of SUR1, a member of the ATP-binding protein superfamily, and $K_{IR}6.2$, a small K^+ inward rectifier ([10–12], reviewed in [7, 13, 14]). All of the available evidence is consistent with $K_{IR}6.2$ forming a tetrameric pore whose activity, in the native channel, is regulated by SUR1. Our current efforts, reviewed here, are focused on understanding how SUR couples with $K_{IR}6.2$ to specify the properties of native K_{ATP} channels including their spontaneous bursting and inhibition by ATP, with an emphasis on the functions of the cytoplasmic domains $K_{IR}6.2$.

Two Segments of SUR Specify the Bursting Pattern and ATP Sensitivity of K_{ATP} Channel Isoforms

Consistent with out previous reports [13, 15, 16] a comparison of kinetics of human β-cell, SUR1/$K_{IR}6.2$, and human cardiac, SUR2A/$K_{IR}6.2$, channels shows that SUR2A/$K_{IR}6.2$ exhibit longer bursts and spend less time in interburst gaps than SUR1/$K_{IR}6.2$ channels while the intraburst kinetics of the two channels are the same (fig. 1). A distinguishing characteristic of the SUR2A/$K_{IR}6.2$ channels is their higher maximal open probability, Po_{max} (0.91 ± 0.01 vs. 0.64 ± 0.03 at –40 mV, respectively). A second distinguishing characteristic is the apparent sensitivity to ATP; the SUR1/$K_{IR}6.2$ channels are approximately 4-fold more sensitive to ATP than the SUR2A/$K_{IR}6.2$ channels with both heteromeric channels being markedly more sensitive than the homomeric $K_{IR}6.2$ channels [17]. In a recent study, we engineered matched pairs of chimeric human SUR1-SUR2A receptors in order to identify the regions of SUR that specify these differences between the β-cell and cardiac


SUR1
K_IR6.2
SUR2A
K_IR6.2
NBF1
NBF2
3 pA
300 ms
Relative NPo
1.0
0.5
0.0
1
10
100
1000
10000
[ATP] (μM)
a
Channel
IC50 (μM)
h
SUR1/K_IR6.2
5.9±0.5
1
SUR2A/K_IR6.2
23.4±2.6
1
K_IR6.2ΔC35
221.1±12.1
1
Relative NPo
1.0
0.5
0.0
1
10
100
1000
10000
[ATP] (μM)
b
Channel
IC50 (μM)
h
SUR2A~SUR1/K_IR6.2
7.8±0.6
1.5±0.2
SUR1~SUR2A/K_IR6.2
31.5±3.1
1.1±0.1

1

channel isoforms [18]. Chimeric receptors were generated by swapping progressively longer segments organized to include the major structural features of SUR. A comparison of one pair of matched chimeric channels in which the SUR N-terminal transmembrane domains were swapped onto the reciprocal C-terminal segments, containing both nucleotide binding folds (NBFs) and the second set of transmembrane domains, is shown in figure 1b. The SUR1 ~ SUR2A/K_{IR}6.2 channels display the bursting of the β-cell channel, but require around 4-fold higher concentration of ATP for half-maximal inhibition, while the matched SUR2A ~ SUR1/K_{IR}6.2 channels display cardiac channel interburst kinetics and the ATP sensitivity expected from β-cell channels (fig. 1b and the analysis in [18]). The intraburst kinetics of the chimeric channels, like the wildtype isoforms, are the same. The analysis of additional chimeras shows that a segment containing the first five transmembrane domains, termed TMDI-N, specifies the higher Po_{MAX} of the cardiac channel, while the lower IC_{50} value for ATP, $IC_{50(ATP)}$, of the β-cell channel is specified by the last 42 amino acids of SUR1 (fig. 2). Note that the C-terminal 45 amino acids is the last exon of SUR2 and that differential usage of two exons specifies the difference between SUR2A and SUR2B.

The results show that two structural elements are critical for determining the isoform dependent components of gating and its modulation by inhibitory ATP and that these elements do not include the nucleotide binding folds of SUR. The observation that a region of SUR can modulate gating raises the question of what segment(s) of K_{IR}6.2 are involved. We have approached this

Fig. 1. SURs specify spontaneous bursting and ATP sensitivity of K_{ATP} channel isoforms. *a* Properties of nonchimeric, wild-type channels. The SUR/K_{IR}6.2 topology maps indicate the receptor/K_{IR}6.2 pairs being analyzed. The swapped segments from SUR1 and SUR2A are shown in gray and black, respectively. The traces are representative example of currents through single channels recorded in the inside-out configuration at –40 mV with $[K^+]_o = [K^+]_i$ ~150 mM [18] illustrating the longer average burst length of cardiac-type SUR2A/K_{IR}6.2 channels. The dose-response curves show the difference in ATP sensitivity of the two channels isoforms; the dose-response of the homomeric K_{IR}6.2ΔC35 channel is shown for comparison [17]. The mean $IC_{50(ATP)} \pm$ error values and a slope factor (h) obtained from the best-fit to a conventional pseudo-Hill equation are given. *b* Properties of one pair of chimeric K_{ATP} channels. The approximate positions of the swapped regions are shown in the topology maps. Examination of currents through single channels shows that the long-bursting characteristic is associated with the N-terminal region of K_{IR}6.2. The Po_{max} values for the SUR2A ~ SUR1/K_{IR}6.2 vs. SUR1 ~ SUR2A/K_{IR}6.2 channels were 0.91 ± 0.01 vs. 0.64 ± 0.03 ($n = 4$ for both channels), respectively. The dose-response data show the lower ATP sensitivity of the β-cell channel is associated with the C-terminus of SUR1. In this and the following figures, downward deflections of the current trace correspond to inward currents, and the horizontal dotted lines show the level of current when all K_{ATP} channels are closed.

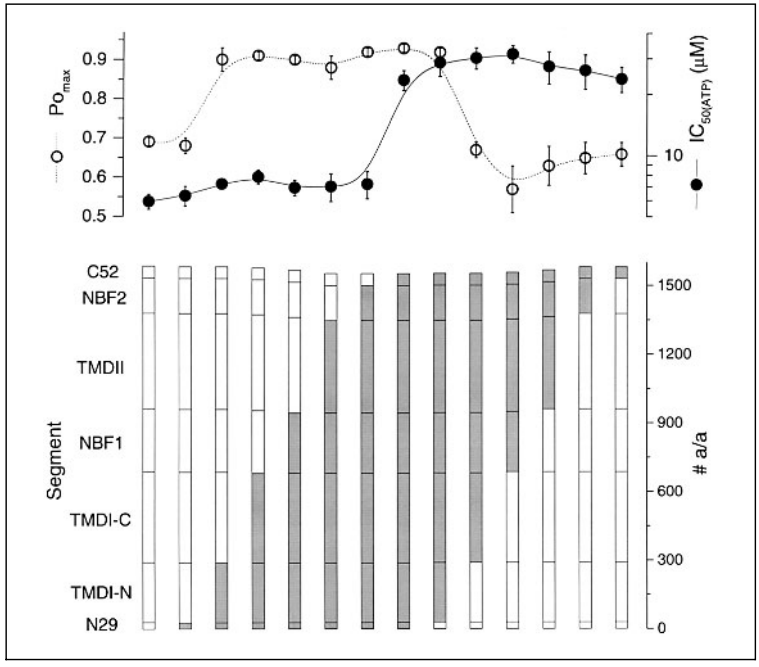


Fig. 2. Delineation of two segments of SUR that specify the differences in kinetics and ATP-inhibition of K_{ATP} channel isoforms. The top panel shows the analysis of the Po_{max} and $IC_{50(ATP)}$ for chimeric channels assembled from wildtype $K_{IR}6.2$ and each of the SUR constructs shown in the lower panel. SUR1 is shown in white, SUR2A in gray. The swap of a C-terminal segment, C52, which changes the last 42 amino acids is sufficient to confer the $IC_{50(ATP)}$ value of the donor SUR isoform. The presence of the TMDI-N segment from SUR2A correlates with a higher Po_{max} value. The Po_{max} values were determined at 0 mV with $[K^+]_o = 5$ mM and $[K^+]_i \sim 150$ mM [18]. Figure adapted from Babenko et al. [18] with permission of the publisher.

issue by modifying the N- and C-termini of $K_{IR}6.2$ to determine their functions in channel gating.

Deletion of the N-Terminus of $K_{IR}6.2$ Results in Channels That Burst Continuously

Coexpression of SUR1 with N-terminally truncated $K_{IR}6.2$, missing 2–32 ($\Delta N32K_{IR}6.2$) or 2–44 ($\Delta N44K_{IR}6.2$) amino acids, produced channels that displayed an unusually high Po_{max} (fig. 3 and reference [19]). Kinetic analysis of single-channel currents shows that the SUR1/$\Delta N32K_{IR}6.2$ and SUR1/

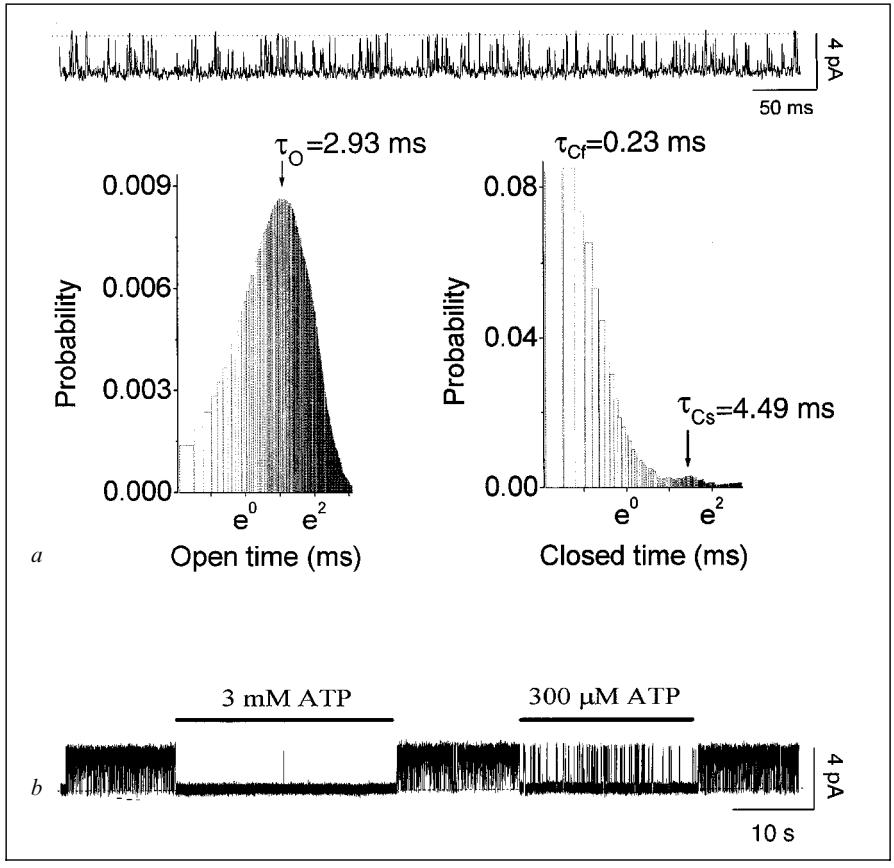


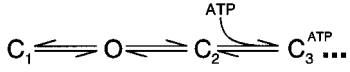
Fig. 3. K_{ATP} channels assembled from $\Delta NK_{IR}6.2$ and SUR1 burst continuously with wildtype intraburst kinetics. *a* Inward currents show extremely long bursts. Currents through a single SUR1/$\Delta N44K_{IR}6.2$ channel recorded and analyzed as described previously [19]. The probabilities of finding a SUR1/$\Delta N32K_{IR}6.2$ channel in the open or closed state are shown. Note that the chance of observing channels with longer closed times is negligible. The time constants for the openings and gaps within a burst (2.93 and 0.23 msec, respectively) are nearly identical to those of the wildtype channel. *b* SUR1/$\Delta NK_{IR}6.2$ channels have a reduced sensitivity to ATP. An example of outward currents through a SUR1/$\Delta N44K_{IR}6.2$ channel showing its attenuated response to inhibitory ATP. At 300 μM ATP, the activity of the wildtype SUR1/$K_{IR}6.2$ channel is practically undetectable.

$\Delta N44K_{IR}6.2$ channels burst nearly continuously with a Po_{max} that is nearly equal to the theoretical limit $\tau_o/(\tau_o + \tau_{Cf})$, for a channel that is in an infinitely long burst and has the same intraburst kinetics as the wildtype channel. A slower component can be seen in the closed time distribution, but this is negligible and consistent with the infrequent interburst closed states. The

burst length and number of openings per burst are on average around 10–100 times higher than we observe (fig. 3 and reference [19]), and have been reported by others [20, 21], for recombinant SUR1/K_{IR}6.2 or β-cell K_{ATP} channels using similar conditions. Thus while deletion of over half of the N-terminus did not affect the intraburst kinetics, it did dramatically decrease the rate of termination of bursting and decrease the fraction of time the channel spends in interburst closed states generally considered to interact with ATP [20, 22, 23].

SUR1/ΔNK$_{IR}$6.2 Channels Have a Reduced Sensitivity to Inhibitory ATP

SUR1ΔNK$_{IR}$6.2 channels are inhibited by ATP with $IC_{50(ATP)}$ values around 20-fold higher than observed for wildtype channels and there is little difference in the ATP sensitivity of the SUR1/ΔN32K$_{IR}$6.2 vs. SUR1/ΔN44K$_{IR}$6.2 channels (106.8 ± 7.8 and 120.9 ± 6.3 vs. 5.9 ± 0.5 μM for the wild-type channel [19]). Note that the N-terminally truncated heteromeric channels are more sensitive to ATP than the homomeric K_{IR}6.2ΔC channels [17]. The moderate increase in the apparent $IC_{50(ATP)}$ values can be explained using a minimal linear kinetic scheme (see (1) and references [20, 22, 23]) without assuming that the N-terminus is part of an ATP-binding site [19]. In this model ATP inhibits channel activity interacting with interburst closed states (C_2). The deletion of the N-terminus which terminates a burst (the $O \rightarrow C_2$ transition) decreases the occupancy of the C_2 state. At a fixed concentration of ATP, and affinity of the C_2 state for the nucleotide, this will reduce the occupancy of the ATP-bound C_3 and consecutive long-lived ATP-bound closed states. Thus a higher concentration of ATP is required to keep the channel closed half of the time giving a higher apparent $IC_{50(ATP)}$ value. The structural basis for control of burst duration is unknown, but the recent work of Perozo et al. [24] and the structure of the bacterial KcsA potassium channel with a two transmembrane helix topology [25] suggests the gate lies near the cytoplasmic end of the M2 helix. This would imply that the N-terminus interacts with a segment of the C-terminal cytoplasmic domain adjacent to the M2 helix. Since the SUR1ΔNK$_{IR}$6.2 channels spend less time in interburst closed states like C_2, the kinetic scheme provides a mechanism to explain the apparent decrease in ATP sensitivity. A similar mechanism would apply to other mutations in K_{IR}6.2 that display parallel increases in Po_{max} and $IC_{50(ATP)}$ [26–29]. There is no compelling reason at present to propose that these mutations



(1)

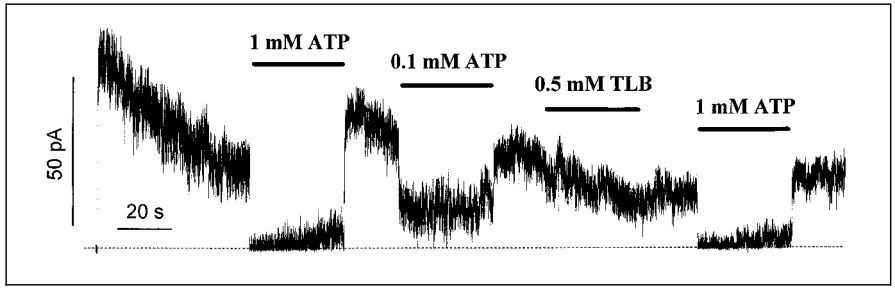


Fig. 4. Deletion of the $K_{IR}6.2$ N-terminus eliminates the sensitivity of the β-cell channel to tolbutamide. An example of macro-currents through SUR1/ΔN44$K_{IR}6.2$ channels. Excision into nucleotide-free media activates K^+ currents that rundown and are reactivated by high concentrations of ATP in the presence of Mg^{2+}. The application of 0.5 mM tolbutamide, sufficient to inhibit a wildtype SUR1/$K_{IR}6.2$ channel has little effect on the SUR1/ΔN32$K_{IR}6.2$ or SUR1/ΔN44$K_{IR}6.2$ channels.

would alter the affinity of the C_2 state for ATP or that the N-terminus is part of an ATP-binding site.

It is tempting to link our observations on the effects of the TMDI-N segment of SUR on channel kinetics with the finding that the N-terminus of $K_{IR}6.2$ limits bursting and speculate these regions interact. A direct physical link has not been established, however, photolabeling experiments suggest these regions are adjacent. Our early biochemical experiments place the site of labeling of SUR1 with ^{125}I-io- doglibenclamide within the first five transmembrane domains [30]. We have shown that ^{125}I-iodoazidoglibenclamide labels both SUR1 and $K_{IR}6.2$. Our unpublished data (N. Sharma and L. Aguilar-Bryan) indicates that deletion of the N-terminus eliminates the labeling of $K_{IR}6.2$, but has no effect on labeling of SUR1. In addition, as shown in figure 4, deletion of the N-terminus eliminates the inhibitory effect of tolbutamide. The results imply the two segments of K_{ATP} channel subunits are in close proximity and suggest the N-terminus is a key element in coupling conformational changes in SUR to the channel pore.

Deletion of the C-Terminus of $K_{IR}6.2$ Allows Surface Expression of Homomeric ($K_{IR}6.2\Delta C)_4$ Channels Weakly Sensitive to ATP

Our detailed examination [17] of currents through single human $K_{IR}6.2\Delta C35$ channels confirms and extends previous observations that deletion of the C-terminus allows expression of channels in the absence of SUR [31]

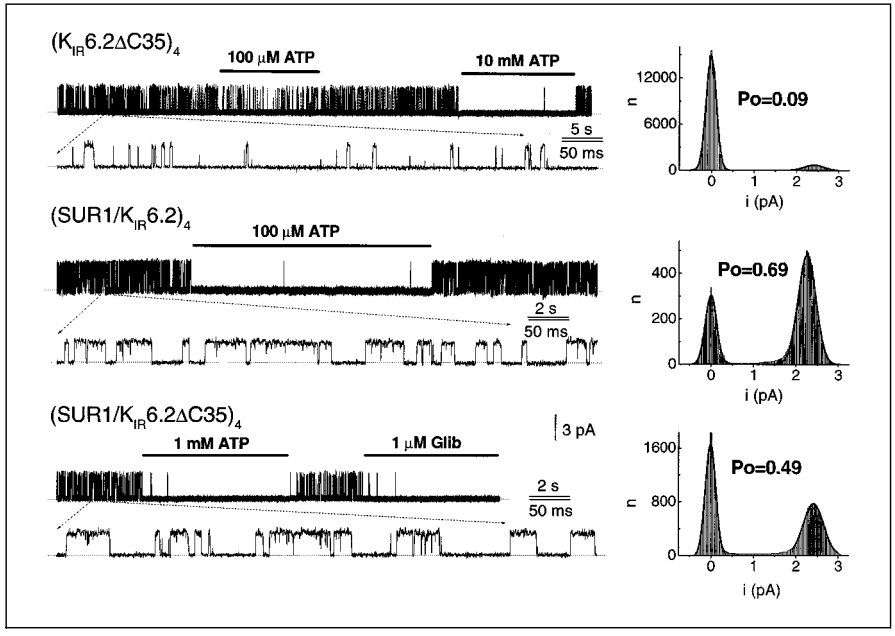


Fig. 5. Homomeric, C-terminally truncated channels show low activity and reduced ATP sensitivity that is restored by coassembly with SUR1. The currents through single $K_{IR}6.2\Delta C35$, SUR1/$K_{IR}6.2$, and SUR1/$K_{IR}6.2\Delta C35$ channels, and the corresponding current amplitude histograms and maximal Po values, are illustrated. Note the marked decrease in burst length when comparing the $K_{IR}6.2\Delta C35$ channels with the SUR1/$K_{IR}6.2$ channels. This characteristic and the apparent sensitivity to ATP are restored by SUR1. Glib = Glibenclamide. Figure from Babenko et al. [17] with permission of the publisher.

whose spontaneous activity is qualitatively different than those from wildtype K_{ATP} channels [29]. The number of openings within a burst is markedly reduced (fig. 5), [17]. Quantitation of their spontaneous bursting indicates the mean burst duration of the homomeric channels is 10–100 times shorter than that of either β-cell of cardiac K_{ATP} channels [16, 20, 21]. The data suggest the homomeric channels leave a burst for a long-lived closed state at a much higher rate than wildtype channels resulting in a markedly lower maximal open probability for the homomeric channels.

Coassembly of $K_{IR}6.2\Delta C35$ with SUR1, verified by showing the currents are glibenclamide sensitive, nearly but not completely restores the Po_{max}, the bursting pattern and sensitivity to inhibitory ATP. In all of the channels tested, including SUR1/$K_{IR}6.2\Delta C35$ and SUR1/$K_{IR}6.2\Delta C35_{K185Q}$, the removal of SUR1 has two major effects: a reduction of the Po_{max} and an increase in the

$IC_{50(ATP)}$ [17]. The markedly higher IC_{50} values observed in the mutant homomeric channels are the result of the additive effects of not having SUR present and of the mutations. In the absence of SUR, $K_{IR}6.2$ spends more time in a long-lived closed state which others have argued binds inhibitory ATP [28, 29] thus, in terms of a linear model (1), the reduced ATP sensitivity of the homomeric channels cannot be explained by reduced occupancy of interburst closed states. SUR, on the other hand, increases the Po_{max} mainly by shortening the interburst interval which, as discussed above, should reduce the apparent $IC_{50(ATP)}$. This negative correlation between Po_{max} and $IC_{50(ATP)}$ implies SUR increases the affinity of interburst closed states for ATP and/or improves the linkage between the binding site and the gate. An alternative possibility is that the ATP-binding site in the heteromeric channel is a shared site between segments of $K_{IR}6.2$ and SUR or another protein.

Analysis of inward currents through single $K_{IR}6.2\Delta C35$ channels (fig. 6) reaffirms the conclusion that the reduced Po_{max} is the result of a marked increase in the percentage of long-lived closures and shows, in contrast to the heteromeric channels assembled from either chimeric receptors or $\Delta NK_{IR}6.2$ subunits, that the mean open time is reduced approximately 2.2-fold in the absence of SUR. The results demonstrate that association with SUR increased the stability of the open state in addition to markedly slowing the rate at which the heteromeric channel leaves a burst. Our interpretation is that allosteric interactions between SUR and $K_{IR}6.2$ are required to convert the poorly operational K_{IR} into a normal high Po_{max} K_{ATP} channel.

We have used the Po_{max} values for the homomeric channels, for heteromeric channels assembled from SUR1 and $K_{IR}6.2\Delta C$, and for wildtype channels (fig. 5) to determine whether coexpression with SUR1 would affect the density of channels in the plasma membrane [17]. The product (NPo) of channel number, N, and the probability of a channel being open, Po, were estimated from ATP-inhibited currents in inside-out patches from identically transfected cells under controlled conditions [17]. The currents from cells expressing SUR1/$K_{IR}6.2\Delta C35$ channels were inhibited equally by ATP and glibenclamide indicating they were through heteromeric channels. Comparison of the relative NPo values, normalized to the wildtype value, indicate SUR1 can increase the relative number of $K_{IR}6.2\Delta C35$-based channels in the surface membrane approximately 7 fold to a density that is around 60% of the wildtype value. The combination of a reduced Po_{max} and reduced surface density accounts for the low homomeric $K_{IR}6.2\Delta C$ channel macro-currents that we observe and have been reported previously [31]. The increased density of channels suggests SUR can either facilitate trafficking to the plasma membrane and/or slow recycling thus increasing the steady-state density. The data are consistent with our initial observation [10] that coexpression of $K_{IR}6.x$ with SUR1 results in

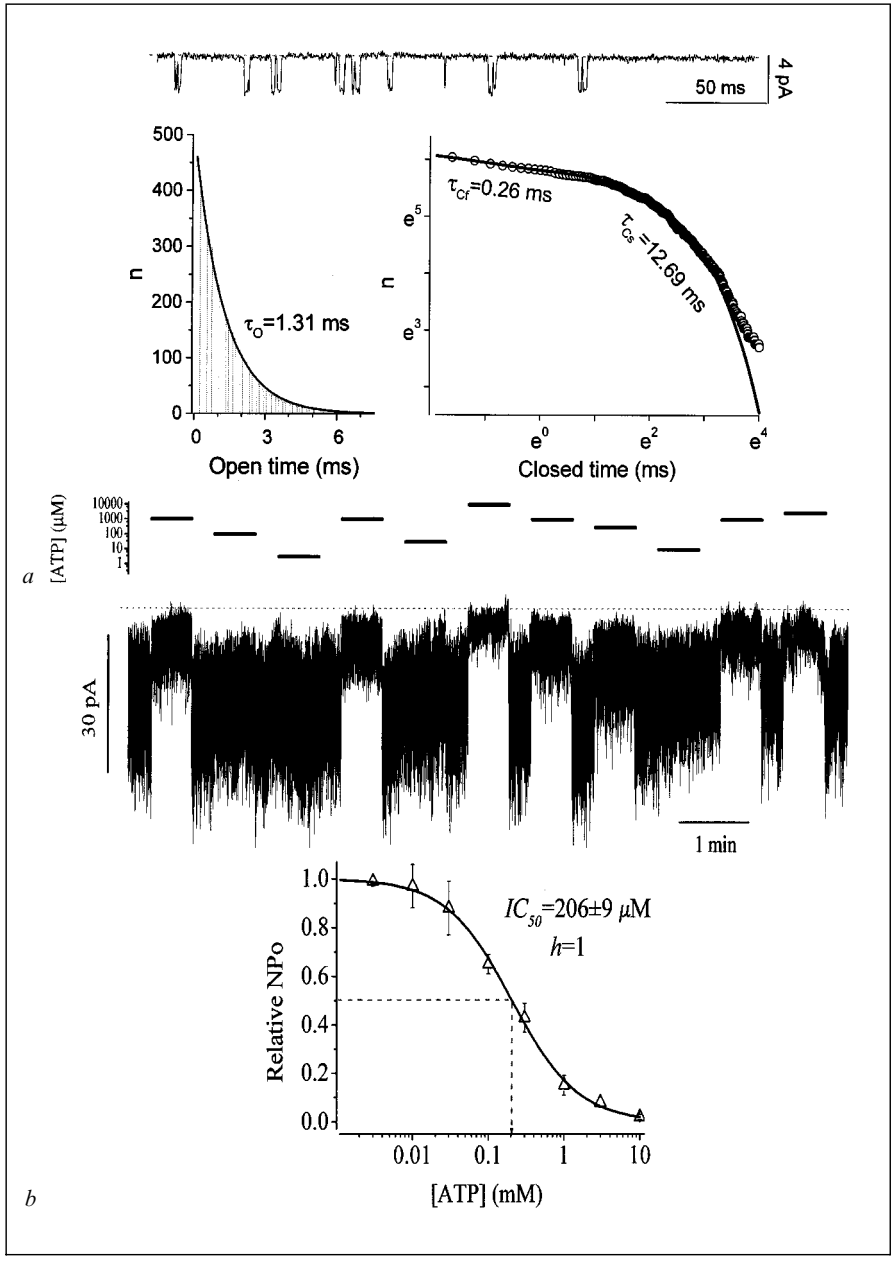


Fig. 6. Single-channel kinetics of homomeric $K_{IR}6.2\Delta C$ channels. *a* Inward currents through a single $K_{IR}6.2\Delta C35$ channel at –40 mV [17]. The open and closed time distributions are given along with the estimated time constants. Note the decrease in τ_O, when compared

the appearance of a mature glycosylated form of the receptor that has passed through the Golgi apparatus, and with the suggestion that the mature receptor associated with $K_{IR}6.2$ makes up K_{ATP} channels in the surface membrane. K^+ currents similar to those of the homomeric $K_{IR}6.2$ channels have not been observed in PHHI β-cells [32] or in the β-cells of SUR1 null mice (Seghers, Nakazaki, Aguilar-Bryan and Bryan, unpublished data), indicating that $K_{IR}6.2$ must traffic poorly to the cell surface in the absence of a functional SUR. This is in complete agreement with the recent discovery of an ER retention signal on the C-terminus of $K_{IR}6.2$ [33]. There is no evidence at present that homomeric $K_{IR}6.2$ channels are of physiological importance and it is not yet understood why Nature has had to devise a quality control mechanism to insure $K_{IR}6.2$ does not reach the cell surface without SUR.

Hunting for an ATP-Binding Site in the C-Terminus of $K_{IR}6.2$

The observation that homomeric K_{ATP} channels retain weak sensitivity to ATP has prompted a search for the nucleotide-binding site on $K_{IR}6.2$ by site-directed mutagenesis. Some mutations in the C-terminal cytoplasmic domain reduce the sensitivity to inhibitory ATP and increase the Po_{max} [27, 29, 31], similar to deletion of the N-terminus, and thus cannot be used as indicators of the location of the ATP-binding site as discussed above. Other mutations, specifically the K185Q substitution in the segment below M2, increases the $IC_{50(ATP)}$ value to >4 mM with little or no effect on channel kinetics making this residue a potential candidate for direct participation in ATP binding [31]. However, it has been impossible to rule out the idea that this segment below M2 forms a link between a more distal binding site and the channel gate and that mutations affect coupling between the two. Drain et al. [29], for example, have implicated a $-F_{333}GNTIK_{338}-$ motif similar to that found in ion-motive ATPases as a candidate ATP-binding site which must be linked to the channel gate. Thus while there is no clear evidence for the location of a nucleotide-binding site on $K_{IR}6.2$, the consensus is that there is a low-affinity ATP-binding site, that it resides in the C-terminal cytoplasmic domain, and that nucleotide binding reduces channel activity.

with the wild-type values given in figure 3. *b* Apparent ATP sensitivity of C-terminally truncated $K_{IR}6.2$ channels. An example of the inhibition of currents through homomeric $K_{IR}2\Delta35$ channels by ATP and the corresponding dose-response curve. The ATP-inhibition analysis was done under steady-state conditions as described [17].

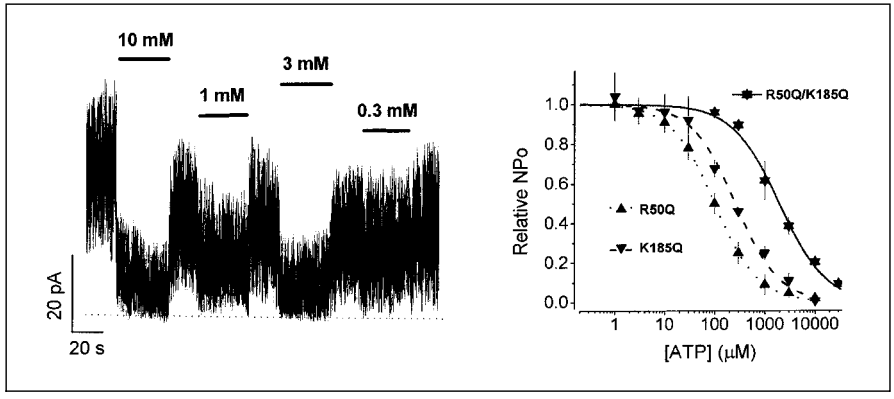


Fig. 7. Combined mutations in the N- and C-terminal cytoplasmic domains of $K_{IR}6.2$ markedly attenuate ATP sensitivity. *a* An example of currents through SUR1/$K_{IR}6.2_{R50Q/K185Q}$ channels and their inhibition by ATP. *b* Corresponding dose-response curves for the SUR1/$K_{IR}6.2_{R50Q/K185Q}$ channel and the individual mutant channels, SUR1/$K_{IR}6.2_{R50Q}$ and SUR1/$_{K185Q}$, respectively. The ATP-inhibition analysis was done under steady-state conditions as described [19].

The N- and C-Termini Cooperate in ATP-Inhibitory Gating

Reduction of the ATP sensitivity by the N- and C-terminal modifications appears to be due to different mechanisms, therefore we asked whether a combination of N- and C-terminal substitutions would have an additive effect on the $IC_{50(ATP)}$ [19]. We engineered substitutions in the N- and C-termini at R50 (R50Q) and K185 (K185Q), positions previously shown to be important for the ATP sensitivity of homomeric $K_{IR}6.2\Delta C$ channels [27, 31] to produce a double mutant channel, SUR1/$K_{IR}6.2_{R50Q/K185Q}$. Individually the R50Q and K185Q mutations produced moderate increases of around 20- and 40-fold in the $IC_{50(ATP)}$ values for the β-cell channels, respectively, without significant effects on the kinetics of heteromeric (0.82 ± 0.01 vs. 0.69 ± 0.01, 0.7 ± 0.01, and 0.68 ± 0.02 for the SUR1/$K_{IR}6.2$, SUR1/$K_{IR}6.2_{R50Q}$, and SUR1/$K_{IR}6.2_{K185Q}$ channels, respectively (at 0 mV with $[K^+]_o = 5$ mM and $[K^+]_i \sim 150$ mM, $n = 4$ for each) and homomeric channels (see also [17, 27]). Note that the effect of the R50Q mutation on $IC_{50(ATP)}$ in the heteromeric channel was significantly greater than the <4-fold decrease observed in $K_{IR}6.2\Delta C26$ channels [34]. The double mutant displayed an increased Po_{max} [19] and reduced the ATP sensitivity dramatically, around 400-fold (fig. 7). The results imply that the cytoplasmic domains of $K_{IR}6.2$ contribute cooperatively to spontaneous bursting and its inhibition by ATP. The additive effect of modifying both the N- and C-termini

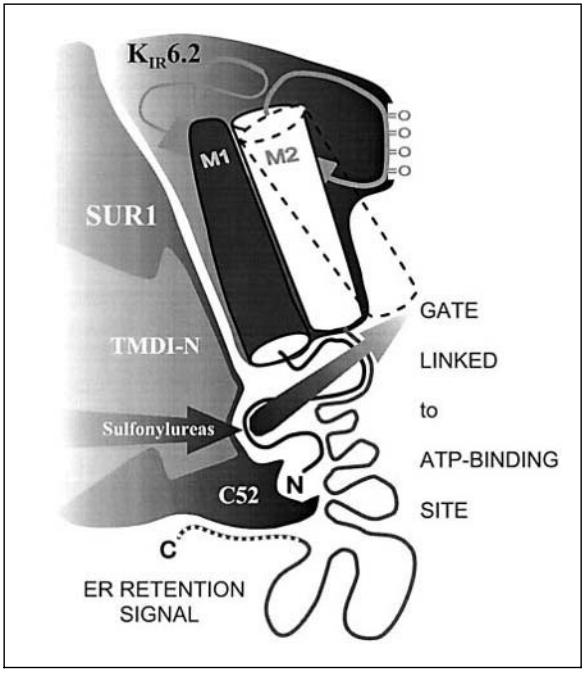


Fig. 8. A summary model for K_{ATP} channel gating. A single pair of subunits within a channel is represented for simplicity. The channel pore, formed by the M2 helix and adjacent C-terminal segment, would lie on the right. The $K_{IR}6.2$ model is based on the structure of the bacteria KcsA K^+ channel [25] and assumes a 'gate' at the bottom of M2. Reorientations of M2 could determine the gating transitions kinetics [24]. K^+ selectivity is conferred by interactions of the backbone carbonyl groups (=O) of the –GFG– motif with K^+ at the extracellular face of the channel [25]. An ER retention signal is indicated on the C-terminus of $K_{IR}6.2$. The N-terminus of $K_{IR}6.2$ is positioned to interact with a segment of the C-terminal cytoplasmic domain that can affect reorientation of M2 to limit bursting. The TMDI-N segment of SUR is positioned near the N-terminus based on our observations that this region of SUR specifies isoform differences in bursting. We propose SUR affects burst duration by coupling to the N-terminus and MI of $K_{IR}6.2$. As discussed in the text, the C-terminal cytoplasmic domain is thought to contain an ATP-binding site linked in some unspecified manner to the gate. Mutations that affect either the affinity of the ATP-binding site or the linkage are expected to alter the $IC_{50(ATP)}$. As described in the text, we propose that sulfonylureas act by affecting the coupling between TMDI-N on SUR1 and the N-terminus of $K_{IR}6.2$. The simplest mechanism consistent with the data suggests that the meglitinide head group of glibenclamide binds near TMDI-N and the N-terminus, and that this binding affects their coupling to lock M2 into a closed orientation as suggested by the arrows.

implies the two cytoplasmic domains of $K_{IR}6.2$ make contributions to ATP-inhibitory gating via distinct mechanisms; the N-terminus by controlling the transition to an ATP-sensitive interburst closed state, the C-terminus by interaction with ATP.

Summary Model

We have tried to fit our data into somewhat speculative model for a K_{ATP} channel. We assume the pore of the channel will resemble the structure of the two-transmembrane helix bacterial K^+ channel [25]. M2 helices line the pore, consistent with mutations in M2 that affect the rectification properties of K_{ATP} channels [10, 26]. The selectivity filter is on the extracellular face and contains the consensus –G(Y/F)G– motif diagnostic of a K^+ channel. The 'gate' is assumed to be near the bottom of M2 and gated access of K^+ to the pore is controlled by movement of the M2 helix as suggested by spectroscopic measurements [24]. There is no data indicating SUR forms part of the pore and we assume it surrounds the $K_{IR}6.2$ tetramer [10, 11, 13]. One SUR1/$K_{IR}6.2$ pair is illustrated schematically in figure 8. Consistent with our finding that the N-terminus limits bursting we propose that it interacts with the C-terminus below M2 where it can affect movement of the helix. Our chimeric channel experiments show that a region within TMDI-N [18] specifies differences in bursting behavior; thus we suggest TMDI-N exerts this effect via a link that includes the N-terminus of $K_{IR}6.2$. The chimeric channel data show the C-terminus of SUR specifies differences in ATP sensitivity and we tentatively suggest this region interacts with a poorly delineated region of the C-terminal region of $K_{IR}6.2$. Within this framework binding of inhibitory ATP must stabilize closed orientations of the M2 helix while the stimulatory effects of potassium channel openers and MgADP, which require association with SUR, antagonize this effect. This model suggests a mechanism of action for sulfonylureas. As described above, glibenclamide derivatives will covalently photolabel SUR1 and when the two are in a complex $K_{IR}6.2$ is also labelled. The site of labelling the SUR1 is near the N-terminus [30], while deletion of 32–44 amino acids from the N-terminus of $K_{IR}6.2$ eliminates the photolabelling of $K_{IR}6.2$ arguing these regions are near to each other. This suggests sulfonylureas may exert their effect through the N-terminus of $K_{IR}6.2$ to restrict opening of the gate. Our observation that SUR1/$\Delta NK_{IR}6.2$ channels are insensitive to high-affinity inhibition by tolbutamide is in agreement with the idea that the N-terminus is involved in coupling the functions of the two K_{ATP} channel subunits.

Acknowledgments

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Joseph Bryan, MD, Departments of Molecular and Cellular Biology and Medicine,
Baylor College of Medicine, Houston TX77030 (USA)
Tel. +1 713 798 4007, Fax +1 713 970 0545, E-Mail jbryan@bcm.tmc.edu

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Metabolic Mechanisms and Diabetes Candidate Genes: Insights Gained from Genetic Engineering with Recombinant Adenoviruses

Hal K. Berman, Khiet Y. Trinh, Robert M. O'Doherty, Peter A. Antinozzi, Rosa Gasa, Christopher B. Newgard

Departments of Biochemistry and Internal Medicine and
Gifford Laboratories for Diabetes Research, University of Texas,
Southwestern Medical Center Dallas, Tex., USA

Normal mechanisms of control of fuel homeostasis are profoundly disrupted in individuals with noninsulin-dependent diabetes mellitus (NIDDM). 'Classical' NIDDM is a disease that develops in middle age, is often associated with obesity, and is characterized by a set of metabolic abnormalities that include insulin resistance, a failure to normally control hepatic glucose production, and β-cell dysfunction. While the disease clearly displays a familial inheritance pattern, its genetic basis is complex and incompletely understood. In fact, NIDDM is currently thought of as a family of genetic diseases. This perception has been shaped in part by important recent studies in which genes involved in a subtype of NIDDM, maturity-onset diabetes of the young (MODY), have been described. As detailed in other articles in this volume, different forms of MODY have been described that involve mutations in the glucokinase gene, or in various transcription factors that are involved in β-cell differentiation and insulin secretion, including PDX-1, hepatocyte nuclear factor (HNF)-1α and β, and HNF-4. As each new gene with a linkage to diabetes is discovered, the mechanism by which it causes the disease must be understood. The purpose of this chapter is to summarize the development of high efficiency adenoviral gene transfer vectors as tools for evaluating the biology of candidate diabetes genes and for gaining insights into basic metabolic control mechanisms.

Recombinant Adenovirus Has Advantages for Metabolic Engineering Studies

There are a wide variety of methods for introducing foreign DNA into mammalian cells. When assessing the metabolic impact or control strength of a particular gene, a major factor in choosing a gene transfer vector is that it must deliver the gene to a majority of cells within a population. Another important consideration is the type of cell that one is trying to transfect. In the case of studies of metabolic regulation, it is desirable to deliver genes to primary cell types that are relevant to control of fuel homeostasis such as the islets of Langerhans or liver cells. Cells with low replicative activity (e.g. isolated islets of Langerhans) are not amenable to stable transfection, and nonviral transfection methods such as Ca_2PO_4 coprecipitation, electroporation, and lipofection generally provide gene transfer efficiencies ranging from 2–50% of cells in a population, not sufficient for studies of metabolic regulation. Fortunately, viral gene transfer vectors have been developed that allow high efficiency gene transfer.

Traditionally, most effort has been focused on retroviral vectors, usually derived from murine leukemia virus (MuLV) [1]. Over time, several limitations of these systems have become apparent. In the context of metabolic engineering, the foremost among these is that cell division is required to achieve retrovirus integration into the genome and transgene expression. Further, the efficiency of gene transfer achieved with retroviral vectors is limited, even in dividing cells. Finally, application of this system for delivery of genes to tissues of intact animals has been limited, in part because it has been difficult to produce viral stocks of sufficiently high titer.

More recently, exciting new vector systems have emerged that are based on lentiviruses, an alternate class of retroviruses that includes human immunodeficiency virus (HIV) [2]. A three plasmid system for preparing recombinant lentiviruses has been developed, and these vectors hold promise for stable gene transfer into cultured cells with low replicative activity [2]. Very recently, vectors have been described in which just 22% of the HIV genome is included, involving none of the pathogenic sequences [3, 4]. These vectors have been used to deliver a reporter gene to liver. The gene was expressed for 22 weeks, but it appears that only 3–4% of liver cells were targeted [4]. In sum, these exciting new vectors show great promise, but some lingering safety issues remain, and in vivo efficacy remains to be established.

Given the limitations of RNA virus vectors, increasing attention has been paid to DNA viruses for gene transfer studies, in particular adeno-associated virus (AAV) and adenovirus. AAV has an advantage compared to RNA viruses in that wild-type AAV integrates specifically at a site in human chromosome

19, as opposed to retroviruses whose sites of integration are random [5]. Unfortunately, many AAV vectors lose their capacity for site-specific integration and are limited with regard to the size of DNA inserts to 4.8 kb or less [6]. Until recently, growth of high titer stocks of AAV was complicated by the requirement for wild-type adenovirus or the use of inefficient packaging cell lines, although better cell lines are now available. For all of these reasons, application of AAV to metabolic research has thus far been limited.

Recombinant adenovirus has a number of attractive features for metabolic applications. So-called first-generation adenovirus vectors are usually derived from human serotypes 2 or 5, and contain deletion in the E1A region, which controls expression of other early viral genes and is required for viral replication [7, 8]. Deletion of just the E1A region allows insert sizes of up to 4.8 kb, but larger inserts can be accommodated in first-generation vectors by deletion of the nonessential E3 gene. While the first-generation adenovirus vectors have shortcomings in terms of their immunogenicity and duration of transgene expression in vivo (see below), they have other very important positive attributes. First, they can transduce a wide range of mammalian cells, including liver cells, islets of Langerhans, muscle cells, and cells of the central nervous system. Second, recombinant adenoviruses can be grown at remarkably high titers (in excess of 10^{12} particles/ml). Third, adenoviral vectors are the most efficient of all the viral vectors, with near 100% gene transfer commonly observed in cultured cells. Fourth, adenovirus vectors effectively transduce nondividing cells, providing advantages relative to the MuLV-based retroviruses. Finally, first generation recombinant adenoviruses are easy to prepare. As detailed elsewhere [9], the approach that we have used with consistent success involves insertion of the gene of interest into a cloning vector called pACCMVV.pLpA that contains 17 map units of adenovirus DNA [10], and cotransfection of 293 cells with this plasmid and a second large vector called JM17 that contains nearly all of the adenovirus genome [11]. The recombinant virus that emerges has its E1A region interrupted by the transgene, causing it to be replication-defective and therefore minimally harmful, while retaining full infectivity.

First-generation adenoviruses allow highly efficient gene transfer to both nondividing and dividing cells, but because the viral DNA is not effectively integrated into genomic DNA [12], gene expression is eventually lost in dividing cells. In nondividing cells, adenovirus transferred genes are expressed for essentially unlimited periods of time [13]. Adenovirus can also be used to transfer genes to tissues and organs in intact animals. Systemic infusion of adenoviral vectors results in highly efficient and preferential gene transfer to liver [14–16]. The preferential targeting of adenovirus-transferred genes to liver is probably explained by the sinusoidal vasculature of that organ, which allows direct contact of viral

particles with hepatocytes, while transfer is limited in other tissues by vascular barriers. A well-documented problem with adenovirus-mediated gene transfer in the setting of the intact animal is the limited duration of transgene expression, with the common experience being a 2- to 3-week window in liver of animals that receive a single systemic dose of the first-generation vector. This is due to an immune response to viral antigens such as E2 and E4 [17–19] and to the expressed transgene [20]. The problem of inflammatory responses to viral antigens was initially addressed by creation of adenovirus vectors lacking specific adenovirus early genes [19], but more recently, 'gutless' vectors, in which all viral genes are deleted in the vectors have also been reported [21, 22]. These improvements have increased the period of in vivo transgene expression from 2–3 weeks to periods of 3 months to 1 year. It should be noted that even a period of 2–3 weeks is often sufficient to assess the acute metabolic impact of a particular gene, and the examples of in vivo engineering provided in this chapter will focus on first-generation vectors.

Use of Recombinant Adenoviruses to Investigate Fuel/Secretion Coupling in Islet β-Cells

Insulin secretion is stimulated from pancreatic islet β-cells in the postprandial state by a variety of nutritional and hormonal secretagogues. Glucose is considered to play a primary role, since most other secretagogues fail to exert their effects at low concentrations of the sugar. Fatty acids and certain amino acids are strong potentiators of the glucose response and are clearly important physiological regulators of insulin secretion. Decades of investigation of the mechanism by which glucose stimulates insulin secretion has yielded a general outline of events, but much remains to be learned [23–25]. Studies on the genetic basis of MODY have served to underscore the importance of gaining a clearer understanding of β-cell function. Thus, the mechanistic link between mutations in the glucokinase gene and hyperglycemia in MODY-2 subjects could be rapidly gleaned thanks to prior studies that had established a rate-determining role for glucokinase in β-cell glucose metabolism and regulation of insulin secretion [23]. Newly discovered forms of MODY (MODY-1, -3, -4) have been shown to involve mutations in β-cell transcription factors, resulting in insulin insufficiency. While part of the β-cell insufficiency phenotype may be related to reduced expression of the insulin gene, there is also evidence to indicate that these transcription factors regulate fuel responsiveness. For example, transgenic mice that are heterozygous for knockout of the HNF-1α gene have impaired glucose-stimulated insulin secretion, which is linked to a proportional decrease in the rate of glucose metabolism [26]. The mechanism

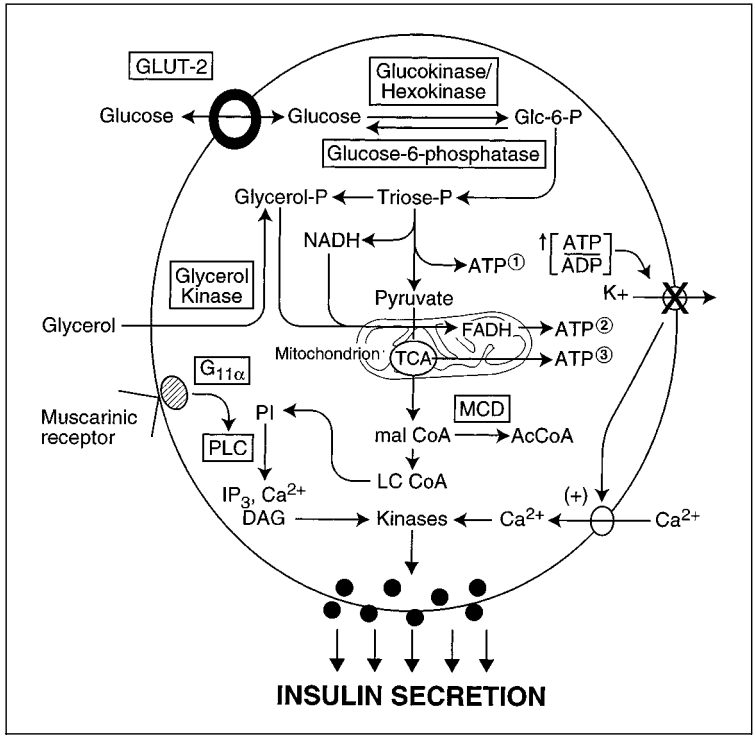

Fig. 1. Stimulus/secretion coupling in the β-cell studied by metabolic engineering. The figure summarizes current concepts of the biochemical mechanism of glucose-stimulated insulin secretion from pancreatic islet β-cells. Key points addressed in this article include: (a) The relative potency of different sites of ATP/ADP metabolism in regulation of K_{ATP} channel activity, including the distal portion of glycolysis (1), the glycerol phosphate shuttle (2), and oxidation of carbohydrate fuels (3); (b) The role of malonyl CoA (mal CoA), long-chain acyl CoA (LC CoA), and phosphoinositide (PI) metabolism in regulation of insulin secretion; (c) Regulation of insulin secretion by levels of expression of glucokinase and its opposing enzyme glucose-6-phosphatase. These issues have been addressed by adenovirus-mediated expression of the genes shown in boxes. Other abbreviations used: Glc-6-P = Glucose-6-phosphate; $G_{11\alpha}$ = heterotrimeric G-protein 11, α-subunit; PLC = phospholipases C; IP_3 = inositol trisphosphate; DAG = diacylglycerol; MCD = malonyl CoA decarboxylase.

by which a decrease in HNF expression is linked to loss of glucose sensing is not known. Uncovering of this linkage could be aided by genetic engineering studies aimed at understanding specific aspects of fuel sensing pathways in the β-cell, such as those described below.

Glucose metabolism is required to cause stimulation of insulin release (fig. 1). Pancreatic islet β-cells contain an ATP-sensitive potassium channel

(K_{ATP}), and stimulation of islets with glucose causes its closure, membrane depolarization, and activation of voltage-gated Ca^{2+} channels. The influx of Ca^{2+} is in turn thought to participate in regulation of protein kinases, possibly including protein kinase C and members of the Ca^{2+}/calmodulin class of kinases, to trigger exocytosis of insulin-containing secretory granules. Further discussion of the experimental evidence supporting this central outline is beyond the scope of this article, but can be obtained in one of several reviews [23–25].

Within this framework lie a number of fundamental and unanswered questions (fig. 1). First, the reactions that are most critical for modulation of ATP:ADP ratio and consequent regulation of the K_{ATP} channel have not been defined. ATP is generated in islets via the distal reactions of glycolysis and by oxidation of pyruvate in the TCA cycle. In addition, islets appear to contain unusually high levels of mitochondrial glycerol phosphate dehydrogenase, which participates in transfer of reducing equivalents from the cytosol to the mitochondria, resulting in production of ATP via FADH and site II of the electron transport chain [27]. Second, a substantial body of correlative evidence suggests that postmitochondrial metabolism of glucose, and regulation of lipid metabolism by glucose, are important for normal regulation of insulin secretion. In fact, exposure of islets to stimulatory glucose has profound effects on lipid metabolism, including suppression of fatty acid oxidation via an increase in malonyl CoA levels [28, 29], and activation of phosphoinositide hydrolysis [30], but the relevance of these events for stimulus/secretion coupling is unclear (fig. 1). In what follows, examples of the use of adenovirus-mediated gene transfer for gaining direct insight into these tissues are provided.

Adenovirus Gene Transfer in Islet Cells

The issues raised above can be addressed by gene transfer experiments. The value of recombinant adenovirus for performing such studies was first appreciated with the demonstration of a 70% gene transfer efficiency in cultured rat islets [13], considerably greater than the 10–20% transfection efficiencies reported previously for the more conventional physical transfection techniques. That viruses can penetrate to the β-cell core was subsequently confirmed by immunofluoresence studies on sectioned islets [31]. Other preparations, such as human islets or islets from Zucker Diabetic Fatty (ZDF) rats are not efficiently transduced by simple coculture with recombinant adenoviruses, perhaps because they are larger and more fibrotic than normal rat islets [32, 33]. Efficient gene transfer can be achieved in ZDF [34] or human [33] islets by perfusing the pancreas with the adenoviral preparation prior to isolating the cells. We have also demonstrated that recombinant adenovirus can be used to deliver genes with near 100% efficiency to insulinoma cell lines such as RIN

1046-38 [35] or INS-1 [36, 37]. Importantly, treatment of normal rat islets [13, 31] or insulinoma cells [36, 37] with a control virus containing the bacterial β-galactosidase gene (AdCMV-βGAL) has no effect on insulin secretion or glucose or lipid metabolism relative to untreated cells, indicating that the virus per se does not interfere with parameters that we wish to analyze in these kinds of experiments.

Overexpression of Glycerol Kinase in β-Cells Provides Insights Into Relevant Sites of ATP:ADP Ratio Changes in Regulation of Insulin Secretion

To gain insight into the relative impact of different sources of ATP and ADP for regulation of K_{ATP} channels, a recombinant adenovirus containing the gene encoding glycerol kinase was constructed [36]. Islet β-cells do not normally metabolize glycerol or respond to it as a secretagogue, but these functions can be conferred by adenovirus-mediated expression of glycerol kinase in isolated rat islets or INS-1 cells [36]. Comparison of the metabolic fates of glucose and glycerol in these cells revealed that a greater proportion of glycerol is converted to lactate and a lesser proportion is oxidized compared to glucose. The two fuels are equally potent as insulin secretagogues, despite the fact that oxidation of glycerol at its maximally effective dose (2–5 m*M*) occurs at a rate that is similar to the rate of glucose oxidation at its basal, nonstimulatory connection (3 m*M*). These findings suggest that ATP produced from mitochondrial oxidation of carbohydrate secretagogues is not the primary signal for insulin secretion. Instead, the acute signal more likely comes from the metabolism of these fuels in the glycerol phosphate shuttle and/or the distal portion of the glycolytic pathway, either of which can lead to production of ATP and an increased ATP:ADP ratio.

That the primary signal may be derived from the distal portion of glycolysis is supported by other recent metabolic engineering experiments. Thus, adenovirus-mediated overexpression of either the mitochondrial or cytosolic forms of glycerol phosphate dehydrogenase (GlyPDH) has no effect on glucose-stimulated insulin secretion in MIN6 or HIT-T15 insulinoma cells, despite a substantial increase in the rate of glycerol metabolism upon overexpression of the mitochondrial form of the enzyme [38]. More recently, the mitochondrial GlyPDH gene has been knocked out in transgenic mice, and animals homozygous for deletion of the gene exhibit normal glucose-stimulated insulin secretion [39]. This finding does not eliminate a role for the glycerol phosphate shuttle in glucose signaling, since the malate-aspartate shuttle is also very active in islet cells [27], and represents an alternative means for transfer of reducing equivalents from the cytosol to the mitochondria. Consistent with this, inhibition of the malate-aspartate shuttle with aminooxyacetate (AOA)

in normal islets has no effect on insulin secretion, but when the drug is applied to islets from mitochondrial GlyPDH knockout mice (resulting in simultaneous suppression of both the glycerol phosphate and malate-aspartate shuttles), glucose-stimulated insulin secretion is completely blocked [38]. Further information about the metabolic status and viability of cells with complete blockade of reducing equivalent shuttles will be required before firm conclusions can be made, including information about the effects of partial as opposed to complete inhibition of these shuttles.

Adenovirus-Mediated Malonyl CoA Decarboxylase Expression as a Test of the Long-Chain Acyl CoA Hypothesis

Another fundamental issue that we have addressed with recombinant adenovirus technology is the importance of the link between glucose and lipid metabolism for regulation of insulin secretion. In recent years the concept that glucose may signal in part via a link to lipid metabolism has received significant experimental support [24, 28, 29, 40]. Stimulation of various β-cell preparations with glucose results in an increase in malonyl CoA levels, resulting in inhibition of fatty acid oxidation. Malonyl-CoA-mediated inhibition of carnitine palmitoyltransferase (CPT I) and fatty acid oxidation diverts long-chain acyl CoA to other metabolic fates, leading Prentki, Corkey and colleagues to propose that these events could be involved in regulation of insulin secretion, an idea now known as the 'long-chain acyl CoA hypothesis' [28, 29]. Metabolic fates of LC-CoA that could be relevant to insulin secretion include conversion to bioactivate metabolites such as diacylglycerol or inositol trisphosphate (IP_3), contribution to plasma membrane or secretory granule membrane lipid turnover, or direct acylation of proteins involved in secretory granule trafficking.

In order to directly test the long-chain acyl CoA hypothesis, we constructed a recombinant adenovirus containing the cDNA encoding malonyl CoA decarboxylase (AdCMV-MCD), an enzyme that decarboxylates malonyl CoA to acetyl CoA [37]. Overexpression of MCD in INS-1 cells caused substantial decreases in intracellular malonyl CoA levels compared to cells treated with AdCMV-βGAL control virus at both 3 and 20 m*M* glucose. Further, at 20 m*M* glucose, AdCMV-MCD-treated cells were less effective at suppressing (1-^{14}C) palmitate oxidation, and incorporated about 50% less labeled palmitate and glucose into cellular lipids than control cells. Overexpression of MCD did not, however, alter the rate of 5-^{3}H glucose utilization or 1-^{14}C glucose oxidation, showing that the molecular strategy chosen was effective at short-circuiting the link between glucose and lipid metabolism without disruption of other metabolic fates of glucose. Despite the substantial perturbation of the normal interaction between glucose and lipids caused by MCD overexpression,

glucose-stimulated insulin secretion was unaltered in these cells compared with untreated or AdCMV-βGAL-treated controls. These findings were corroborated by use of a pharmacologic agent, triacsin C, an inhibitor of long-chain acyl CoA synthase [37]. Administration of the drug to INS-1 cells cause potent attenuation of palmitate oxidation, a sharp reduction in glucose or palmitate incorporation into cellular lipids, and a 47% decrease in total LC-CoA levels, but had no effect on insulin secretion in response to glucose. It should be noted that the foregoing work with AdCMV-MCD and triacsin C was carried out in INS-1 insulinoma cells, which exhibit only a 2- to 4-fold increase in insulin secretion in response to glucose, compared to 5- to 15-fold responses that occur in freshly isolated rat islets. To address this point, triacsin C was also used in studies with freshly isolated rat islets of Langerhans. In such cells, the drug caused the expected suppression of fatty acid oxidation, but again without influencing glucose-stimulated insulin secretion [37]. Finally, incubation of INS-1 cells engineered for glycerol kinase expression with triacsin C caused a near-complete block of incorporation of radiolabeled glycerol into cellular lipids, but had no effect on glycerol-stimulated insulin secretion [36].

In light of these results, the following modification of the long-chain acyl CoA hypothesis has been suggested [37]. Our studies employing recombinant adenoviruses and triacsin C show that significant impairment of the link between glucose and lipid metabolism in β-cells is tolerated with no impact on glucose-stimulated insulin secretion. Importantly, glycolytic flux was not affected by treatment of β-cells with AdCMV-MCD or triacsin C. In terms of the ongoing search for metabolic coupling factors that mediate glucose-stimulated insulin secretion, both the MCD and glycerol kinase studies call attention to immediate byproducts of glucose catabolism, particularly reactions of the distal portion of glycolysis and/or the glycerol phosphate shuttle. It is important to emphasize, however, that these studies do not rule out the role for lipids in regulation of insulin secretion. In fact, depletion of islet lipids by administration of nicotinic acid [41, 42], or infusion of a recombinant adenovirus containing the leptin cDNA to normal rats [43], results in complete loss of insulin secretion not only in response to glucose, but also to arginine, leucine, or the sulfonylurea, glibenclamide [43, 44]. Arginine is thought to exert its secretory effects by directly affecting membrane polarization, while sulfonylureas are believed to bring about the same effect by inhibiting ATP-sensitive K^+ channel activity. Thus, both agents act distal to any anticipated early metabolic signal. Yet with all secretagogues tested to date, the attenuated insulin response in lipid-depleted islets is restored to normal by inclusion of fatty acids in the perfusate [41–44]. The mechanism by which fatty acids exert these important modulatory effects on insulin secretion remain to be established.

The Link Between Phophoinositide Metabolism and Insulin Secretion Investigated by Overexpression of Phopholipase C Isoforms and G-Proteins

One possible role for lipids in insulin secretion is their further metabolism to generate byproducts with signaling properties. For example, it has been suggested that insulin secretion from pancreatic islets may be mediated in part by activation of phospholipases C (PLC) and phosphoinositide hydrolysis [30]. PLC-mediated hydrolysis of phosphoinositides would result in generation of inositol trisphosphate (IP_3) and diacylglycerol, which could link to insulin secretion via mobilization of intracellular Ca^{2+} stores and activation of protein kinase C, respectively (fig. 1). To investigate this issue further, we have characterized PLC isozyme expression in normal rat islets and two insulinoma cells lines, INS-1 and βG 40/110 [45]. βG 40/110 is a glucose-responsive cell line derived from RIN 1046-38 insulinoma cells by stable transfection with plasmids encoding human insulin and glucokinase [46, 47]. We find that rat islets contain abundant PLC δ1 expression, but that both cell lines completely lack this isoform. In addition, both lines have similar or slightly reduced levels of expression of PLC β1, β2, β3, δ2 and γ1 as found in fresh rat islets. To determine whether an increase in inositol phosphate (IP) levels will result in enhanced insulin secretion, recombinant adenoviruses containing the cDNAs encoding PLC δ1 β1, or β3 were constructed and used to overexpress all three isoforms in INS-1 or βG 40/110 cells [45]. In these cell lines, overexpression of PLC isoforms resulted in little or no enhancement in IP accumulation and no improvement in insulin secretion in response to glucose or carbachol, despite the fact that the overexpressed proteins were fully active in cell extracts. Overexpression of the β1 or β3 isoforms in normal rat islets elicited a larger increase in IP accumulation, but again with no effect on insulin secretion. Since the effect of carbachol on insulin secretion is thought to be mediated through muscarinic receptors that link that $G_{q/11}$ class of heterotrimeric G-proteins, we also overexpressed G_{11a} in INS-1 cells, either alone or in concert with overexpression of PLC β1 or β3 [45]. Overexpression of G_{11a} enhanced IP accumulation, an effect slightly potentiated by co-overexpression of PLC β1 or β3, but again, these maneuvers were without effect on glucose or carbachol-stimulated insulin secretion. In sum, our studies show a lack of correlation between IP accumulation and insulin secretion in INS-1 cells, βG 40/110 cells, or cultured rat islets during stimulation with glucose or carbachol. However, it remains possible that the loss of fuel signaling engendered by complete lipid depletion in islets [41–43] could be due to a requirement for some minimal level of PLC-mediated phosphoinositide metabolism. Clearly, further studies focused at understanding why intracellular lipids are required for normal regulation of insulin secretion are called for.

Investigation of the Potential Role of Glucose-6-Phosphatase in β-Cell Dysfunction Using Adenovirus Technology

The terminal step of gluconeogenesis is the hydrolysis of glucose-6-phosphate (G6P) to free glucose, catalyzed by the glucose-6-phosphatase enzyme complex. The complex is comprised of a catalytic subunit sequestered within the endoplasmic reticulum (ER), a glucose-6-phosphate translocase known as T1 that delivers glucose-6-phosphate to the catalytic subunit, and putative ER glucose and inorganic phosphate transporters (T2, T3) that move the reaction products back into the cytosol [48, 49]. An increase in activity of the glucose-6-phosphatase (G6Pase) complex has been implicated in β-cell dysfunction of NIDDM. Measurement of glucose formation and utilization in islets of ob/ob mice, a model of obesity and NIDDM, revealed a dramatically increased rate of glucose recycling relative to islets from normal lean animals [50, 51]. The authors suggested that increased glucose cycling could be explained by increased activity of G6Pase, although evidence for this was not presented. Other studies have shown that the mRNA encoding the catalytic subunit of G6Pase is increased by approximately 5-fold in islets of Zucker diabetic fatty (ZDF) rats relative to lean controls [52]. Like islets from ob/ob mice, those from ZDF rats have impaired insulin secretion [52–54]. However, these studies did not directly determine whether an increase in G6Pase catalytic subunit mRNA expression is sufficient to cause impairment of glucose-stimulated insulin secretion.

To directly address the potential role of glucose-6-phosphatase in β-cell dysfunction, recombinant adenovirus was used to overexpress the glucose-6-phosphatase catalytic subunit (G6Pase) in the well-differentiated INS-1 insulinoma cell line [55]. The overexpressed catalytic subunit was normally glycosylated, correctly sorted to the ER, and caused a 4-fold increase in G6P hydrolysis. Overexpression of the catalytic subunit also caused a 32% decrease in glycolytic rate relative to controls, and a proportional decrease in glucose-stimulated insulin secretion. These studies show that overexpression of the G6Pase catalytic subunit alone significantly impacts glucose metabolism and insulin secretion in islet β-cells, without a requirement for overexpression of other components of the hydrolytic complex [55]. However, INS-1 cells treated with AdCMV-G6Pase do not exhibit the severe alterations of β-cell function and metabolism seen in islets from rodent models of obesity and NIDDM, suggesting the involvement of genes in addition to the catalytic subunit of G6Pase in etiology of such β-cell dysfunction.

Metabolic Control Mechanisms in Liver Cells

In mammalian organisms, the liver plays a critical role in control of fuel homeostasis. In individuals with NIDDM, ingestion of a meal, even one

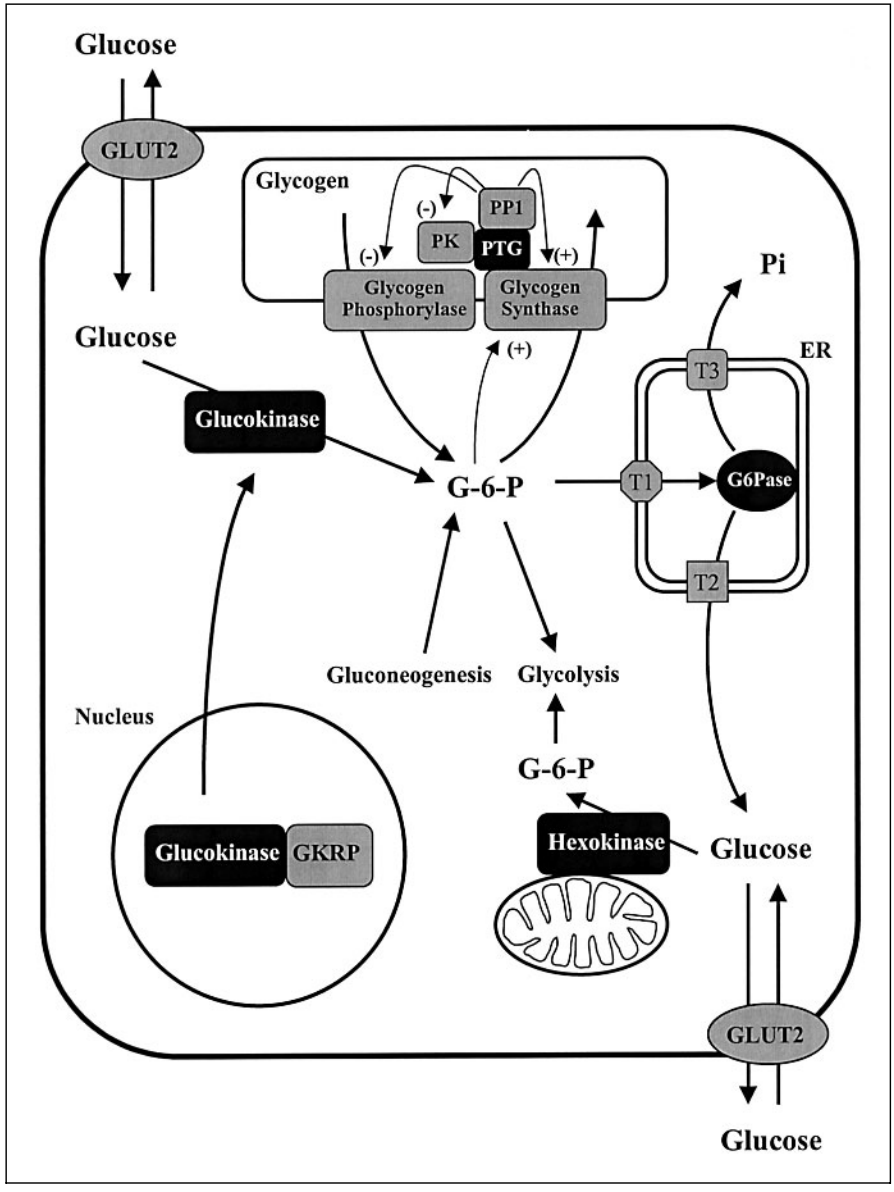

Fig. 2. Metabolic regulation in liver cells studied by genetic engineering. The figure shows some of the key regulatory steps controlling hepatic glucose disposal and production, and emphasizes how these steps are compartmentalized. Examples of this include translocation of glucokinase from the nucleus to the cytoplasm in response to nutritional stimulation, localization and regulation of enzymes of glycogen metabolism via members of the protein targeting to glycogen (PTG) gene family, sequestration of the glucose-6-phosphatase catalytic

containing carbohydrates, fails to exert its normal suppressive effect on hepatic glucose output, and also fails to trigger the normal surge in glucose disposal and storage. NIDDM patients store less glycogen in liver than normal subjects [56] and also can exhibit profound circulating hyperlipidemia as a consequence of peripheral insulin resistance and sustained VLDL production. The rapid increase in incidence of NIDDM in Western society has brought renewed focus on regulation of these fundamental metabolic pathways with the hope that better understanding can be translated into new genetic or pharmacologic therapeutic strategies. In addition, mutations in genes linked to MODY that affect β-cell function such as glucokinase and the hepatocyte nuclear factors (HNFs) are also likely to impact fuel homeostasis through effects on hepatic metabolic function. The following section therefore seeks to summarize the application of adenovirus-mediated gene transfer for understanding of fundamental metabolic control mechanisms in liver.

The Role of Glucose Phosphorylating Enzymes

The balance between hepatic glucose production on the one hand and glucose disposal and storage on the other is determined by the relative rates of glucose phosphorylation and glucose-6-phosphate hydrolysis (fig. 2). Glucose-6-phosphate hydrolysis is mediated by the glucose-6-phosphatase enzyme complex described earlier. Glucose phosphorylation in liver is primarily catalyzed by glucokinase (hexokinase IV). This enzyme has a lower affinity for glucose and a higher catalytic capacity than other members of its gene family, and is limited in terms of its tissue distribution to liver, the islets of Langerhans, and certain specialized neuroendocrine cells in the pituitary and gastrointestinal tract [57, 58].

We have used recombinant adenovirus to compare the metabolic impact of overexpression of glucokinase and hexokinase I in primary rat hepatocytes [59]. Hexokinase I has a much lower Km for glucose than glucokinase (50 μ*M* versus 8 m*M*, respectively), and is also distinct in that it is allosterically inhibited by glucose-6-phosphate [60]. AdCMV-GK and AdCMV-HKI viruses were applied to cultured rat hepatocytes to cause an approximate 7-fold increase in total glucose phosphorylation capacity in each case [59]. A remarkable discrepancy between the metabolic impact of the two hexokinases was ob-

subunit (G6Pase) in the endoplasmic reticulum (ER), and binding of low Km hexokinases to the mitochondria. The metabolic effects of genes shown with black highlighting have been addressed in our laboratory by adenovirus-mediated gene transfer into hepatocytes or liver of whole animals. Other abbreviations used: PP1 = Protein phosphatase-1; PK = phosphorylase kinase; T1, T2, T3 = components of the glucose-6-phosphatase enzyme complex serving as translocases for glucose-6-phosphate, glucose and inorganic phosphate, respectively; GKRP = glucokinase regulatory protein; G-6-P = glucose-6-phosphate.

served, in that AdCMV-GK-treated hepatocytes synthesized 15 times as much glycogen as control cells, while AdCMV-HKI-treated hepatocytes exhibited no increase in glycogen levels. Similarly, AdCMV-GK treatment caused a 3-fold increase in lactate output and a 3.5-fold increase in glucose oxidation, while treatment with AdCMV-HKI caused only a modest increase in lactate output, and then only at low glucose concentrations. Subsequent studies revealed that glucokinase overexpression effectively stimulates glycogen synthase activation, while hexokinase I overexpression fails to do so [61]. These findings may be related to compartmentalization of key enzymes within hepatocytes (fig. 2). In liver, a large percentage of hexokinase I is associated with mitochondria, while glucokinase is found in the nucleus in the presence of low nutrient levels, where it is bound to the glucokinase regulatory protein, and translocates to the cytosol in response to various carbohydrates [62–65]. Overexpressed hexokinase I is efficiently sorted to mitochondria, while overexpressed glucokinase never appears in the mitochondrial fraction [59]. Nuclear binding of glucokinase becomes saturated when the enzyme is overexpressed at levels greater than 2.5 times the endogenous activity [66]. Based on these and other observations, we have proposed that the greater capacity of glucokinase to stimulate glycogen synthase activation may be related to interaction or juxtaposition of the two enzymes within hepatocytes [61]. These enzymes may interact via glucose-6-phosphate, the product of the glucokinase reaction and a known allosteric activator of glycogen synthase.

Glucokinase Overexpression Reveals Fundamental Metabolic Differences Between Liver and Islet Cells

Metabolic engineering with recombinant adenoviruses has also been used to reveal fundamental differences in regulation of glucose metabolism in hepatocytes and islet β-cells. Many years of biochemical investigation have implicated glucokinase as a key enzyme in the control of glucose flux and glucose-stimulated insulin secretion in islets [23–25, 67]. Strong support for this concept also has been gained by the identification of glucokinase as the mutated gene in MODY-2 [68]. Further, experimental reduction in glucokinase expression in liver or islets has been achieved in transgenic mice, resulting in reduced glucose-stimulated insulin secretion and less efficient suppression of hepatic glucose output during glucose clamping [69–71]. Based on these studies of the effects of underexpression of glucokinase, one might have predicted that the overexpression of the enzyme would provide a similar enhancement of glucose utilization in islet cells as was observed in the experiments described earlier in hepatocytes. Surprisingly, adenovirus-mediated overexpression of glucokinase in isolated rat islets resulted in no detectable increase in glucose usage, lactate production, or glycogen synthesis, while overexpression of hexo-

kinase I caused increases in glucose usage and insulin secretion at low glucose concentrations [13, 31].

The well-differentiated insulinoma cell line INS-1 was used for comparison with rat hepatocytes to allow more detailed investigation of the differential impact of overexpressed glucokinase in the two cell types [72]. Overexpression of glucokinase caused a substantial increase in 2- or 5-^{3}H glucose usage at low (≤ 5 mM) but not high glucose concentrations in INS-1 cells, while glucose usage dramatically increased at both low and high glucose concentrations in similarly treated hepatocytes. Further, glucose usage was suppressed in glucokinase overexpressing INS-1 cells in a rapid, glucose concentration, and reversible fashion, while such regulation was largely absent in hepatocytes. Levels of hexose phosphates were profoundly and rapidly elevated following a switch from low to high glucose in glucokinase overexpressing INS-1 cells or hepatocytes relative to controls. In contrast, triose phosphate levels (glyceraldehyde-3-phosphate + dihydroxyacetone phosphate) were much higher in AdCMV-GK-treated INS-1 cells than in similarly treated hepatocytes, suggesting limited flux throughout the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) step in the former cells. AdCMV-GK-treated INS-1 cells had a much higher pyruvate:lactate ratio than in similarly treated hepatocytes [72]. Since the amounts of G3PDH activity in INS-1 and hepatocyte extracts were shown to be similar, it was suggested that flux through this step in INS-1 cells is limited by failure to regenerate NAD in the LDH reaction, and that a fundamental difference between hepatocytes and islet β-cells is the limited capacity of the latter to metabolize glycolytic intermediates beyond the G3PDH step. These findings are generally consistent with those of another group who studied INS-1 cells stably transfected with tetracyclin regulatable glucokinase construct. These authors showed that 2-fold overexpression of glucokinase caused a proportional increase in 5-[^{3}H] glucose usage at both low and high glucose, but that further increases in expression resulted in little additional increase in glycolysis at higher glucose concentrations [73].

The foregoing studies may help to explain recent observations of Polonsky and coworkers in heterozygous glucokinase knockout (GK (+/–)) and normal (GK (+/+)) mice [74]. Culture of GK (+/–) islets at high glucose resulted in increased glucokinase expression and near normalization of impaired glucose-stimulated insulin secretion. Culture of GK (+/+) islets at high glucose concentrations also increased glucokinase expression, but in these islets the increased GK activity was accompanied by increased basal insulin secretion and a loss of the normal incremental response to stimulatory glucose. These findings correlate well with the increase in glucose metabolism at low but not high glucose concentrations observed in glucokinase overexpressing INS-1 cells [72, 73].

Is Hepatic Glucokinase Overexpression a Therapeutic Approach to Treatment of NIDDM?

From a practical perspective, these studies suggest that increasing glucokinase enzymatic activity in liver, but not islets, could serve as a means to lower circulating glucose levels in diabetes. Indeed, in one study, near-normalization of blood glucose levels was achieved in streptozotocin-diabetic mice transgenic for glucokinase expression under control of the PEPCK promoter [75]. Surprisingly, the high levels of fatty acids, triglycerides, and ketone bodies found in streptozotocin diabetes were normalized in the transgenic mice. However, it should be noted that these animals are completely insulin-deficient, making it difficult to predict the metabolic impact of increased glucokinase expression in the presence of the hormone, as would be the case in type 1 patients receiving insulin therapy or in patients with type 2 diabetes. Also, the findings in glucokinase transgenic mice are in contrast to those of our recent study in which we used the AdCMV-GKL adenovirus to achieve overexpression of the enzyme in liver of normal rats [76]. AdCMV-GKL-treated animals had an average increase in glucokinase activity of 6.4-fold relative to rats treated with a virus encoding a catalytically inactive point mutant of glucokinase originally identified in a MODY-2 patient (AdCMV-GK_{203}). Overexpression of glucokinase to this degree resulted in a 38% decrease in blood glucose levels and a 67% decline in circulating insulin levels. However, the decrease in glucose levels came at the expense of a 190% increase in circulating triglycerides and a 310% increase in circulating free fatty acids. Thus, in normal animals, levels of glucokinase overexpression associated with a decline in blood glucose are accompanied by equally dramatic increases in circulating lipids, raising concerns about manipulation of glucokinase activity as a viable strategy for treatment of diabetes. More in-depth testing of the effect of hepatic glucokinase overexpression on glucose and lipid homeostasis is called for, particularly in animal models of diabetes. Since many of these models are very difficult to access by germline manipulation, this is an area in which recombinant adenovirus can continue to provide important insights.

Protein Phosphatase-1 Targeting Proteins and Compartmental Control of Glycogen Metabolism

In light of the potential complications associated with glucokinase overexpression just discussed, we have recently used recombinant adenovirus to uncover a potential new strategy for enhancing hepatic glucose utilization in diabetic patients [77]. Our approach has been to overexpress a recently discovered gene encoding protein targeting to glycogen (PTG), also known as PPP1R5 [78, 79]. PTG is a member of a gene family known collectively as glycogen targeting subunits of protein phosphatase-1 (PP-1) [80]. In addition

to PTG, which is expressed in a wide variety of tissues, the family includes an isoform that is expressed primarily in liver called G_L [81] and another form that is expressed primarily in skeletal muscle and heart known as G_M or RG1 [82]. These proteins target PP-1, the enzyme responsible for activation of glycogen synthase and inactivation of glycogen phosphorylase and phosphorylase kinase [83], to the glycogen particle. They also appear to participate in assembly and regulation of the enzymes of glycogen metabolism within mammalian cells. Thus, in addition to its capacity to bind PP-1 and the glycogen particle, PTG appears to bind directly to glycogen synthase and phosphorylase kinase [78, 79].

A recombinant adenovirus containing the mouse PTG cDNA (AdCMV-PTG) was used to overexpress the protein in primary rat hepatocytes [77], allowing evaluation of its metabolic impact in a cell type that is known to have a large glycogenic capacity. Overexpression of PTG in hepatocytes isolated from fasted rats resulted in potent activation of glycogen synthesis, such that levels of glycogen in AdCMV-PTG-treated cells approximated those in liver of fed animals. Surprisingly, the glycogenic effect of PTG was observed even in the complete absence of carbohydrates or insulin in the culture medium [77]. PTG overexpression also prevented the normal glycogenolytic action of agents such as forskolin or glucagon. These metabolic effects of PTG overexpression were accompanied and likely explained by a 3.6-fold increase in glycogen synthase activation state, and a 40% decrease in glycogen phosphorylase activity. These results are consistent with a model in which PTG overexpression 'locks' the hepatocyte in a glycogenic mode, presumably via its ability to promote interaction of enzymes of glycogen metabolism with PP-1.

These in vitro studies suggest that alteration of expression levels of PTG or its family members may have relevance for treatment of diabetes. A fundamental metabolic defect of NIDDM is a failure to suppress hepatic glucose output in the fed state, and this is reflected in lower levels of hepatic glycogen in individuals with the disease [56]. Upregulation of expression of glycogen targeting subunit proteins in liver by pharmaceutical or genetic methods might serve to enhance glucose disposal and glycogen storage in NIDDM subjects, potentially without exacerbation of hyperlipidemia. However, among the isoforms of glycogen targeting subunits, PTG may not be the optimal candidate for manipulation, since it appears to override normal hormonal control mechanisms for glycogen metabolism. Expression of other family members, such as G_M or G_L, which are regulated by phosphorylation/dephosphorylation and by direct binding to phosphorylase a, respectively, may stimulate glucose disposal while still allowing substrate- and hormone-mediated regulation of glycogen turnover. Construction of recombinant adenoviruses containing the genes for G_M and G_L should allow rapid testing of this idea.

Investigation of the Potential Role of Glucose-6-Phosphatase in Dysregulated Hepatic Glucose Output in NIDDM

It has been suggested that upregulated expression of the catalytic subunit of glucose-6-phosphatase is involved in loss of control of hepatic glucose production characteristic of NIDDM. Indeed, expression of the gene is increased in liver rodent models of both type 1 and type 2 diabetes [84–86]. In the case of the ZDF rat, G6Pase protein and enzymatic activity is increased by approximately 2.5 fold relative to lean ZDF Wistar controls [85]. Further, it has also been demonstrated that metabolic variables associated with diabetes such as hyperglycemia and hyperlipidemia cause increased expression of hepatic G6Pase both in vitro and in whole animal studies [87–89]. Linkage between mutations in the gene encoding G6Pase and human NIDDM have not yet been established. Such an analysis is complicated by the fact that the anticipated lesion is likely to involve enhanced rather than decreased expression of the enzyme, and as such may not involve point mutations or deletions in the protein-coding sequence of the gene. While the search for such genetic mutations continues, further insight into the potential role of G6Pase in the etiology of NIDDM may be gained by studies in which the enzyme is specifically overexpressed in liver.

To this end, recombinant adenovirus has been used to overexpress the catalytic subunit of glucose-6-phosphatase in isolated hepatocytes and in liver of normal animals [85, 90]. Similar to observations in INS-1 cells, overexpression of G6Pase in rat hepatocytes caused a potent (8-fold) increase in G6P hydrolysis and an attendant 25% reduction in intracellular G6P levels [90]. Overexpression of the enzyme in hepatocytes also caused substantial decreases is glycolytic flux and glycogen deposition, and a parallel increase in gluconeogenesis. These studies suggested that upregulated expression of the glucose-6-phosphatase catalytic subunit in liver cells has the potential to perturb fuel homeostasis in whole animals.

To further investigate this point, the recombinant adenovirus encoding the glucose-6-phosphatase catalytic subunit was used to cause a 2- to 3-fold increase in enzyme activity in liver of normal rats. AdCMV-G6Pase-infused rats exhibited several of the abnormalities associated with early-stage NIDDM, including glucose intolerance, hyperinsulinemia, decreased hepatic glycogen content, and increased peripheral (muscle) triglyceride stores. These animals also exhibited significant decreases in circulating free fatty acids and triglycerides, changes not normally associated with the disease.

These findings are consistent with the idea that upregulation of G6Pase expression in liver can make a significant contribution to the development of NIDDM. One possibility would be that G6Pase mutations are the primary genetic lesion in some individuals with the disease. In this scenario, a gradual

rise of G6Ppase overexpression results in development of glucose intolerance and hyperinsulinemia, resulting in turn in increased lipid storage in peripheral tissues, as demonstrated in the study summarized above. Peripheral overstorage of fat is strongly correlated with development of insulin resistance [reviewed in 91]. In the event that lipid overstorage actually causes insulin resistance, one might predict that long-term overexpression of G6Pase in the liver could result in gradual development of hyperlipidemia and exacerbation of glucose intolerance, complementing hyperinsulinemia and reduced hepatic glycogen levels to produce the entire syndrome of NIDDM. Alternatively, overexpression of G6Pase in liver could be secondary to increases in circulating glucose and lipid levels that occur in response to genetic lesions in insulin signaling or β-cell function rather than in the G6Pase gene itself. Finally, intermediate models, in which G6Pase interacts in different ways with other candidate genes that predispose to insulin resistance or β-cell failure can also be contemplated. Adenovirus-mediated delivery of G6Pase to liver of various experimental models of insulin resistance may be used in the future to gain new insights into the interaction of G6Pase overexpression with other genetic factors predisposing to NIDDM.

Conclusions and Future Directions

In this chapter, we have attempted to review recent applications of viral gene transfer technologies to diabetes research. We have focused on recombinant adenovirus as the vehicle of choice, on the basis of the ease of construction of these viruses, the ability to produce high titer stocks, the very high efficiency of gene transfer to relevant primary cell types such as hepatocytes and islets of Langerhans, and the ability to use these vectors for efficient gene delivery to liver of intact animals, an important organ of fuel homeostasis. This technology allows us to manipulate specific steps in metabolic pathways to determine their importance in control of fuel metabolism. They also allow us to evaluate the function of new or disease-related genes in both the in vitro and in vivo setting. Finally, recombinant adenovirus can be used to facilitate chronic delivery of hormones such as leptin in whole animals, thereby allowing new insights into mechanism of action of such agents [92, 93]. The examples provided in this article of the application of adenoviral technology will hopefully serve as a model for testing of genes linked to various forms of diabetes, including MODY, as these emerge. It is also hoped that these approaches may translate into development of novel pharmacologic or gene therapeutic strategies for treatment of metabolic diseases in the future.

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Christopher B. Newgard, PhD, Gifford Laboratories for Diabetes Research,
Room Y8.212, University of Texas Southwestern Medical Center,
5323 Harry Hines Blvd., Dallas, TX 75235 (USA)
Tel. +1 214 648 2930, Fax +1 214 648 9191, E-Mail newgard@utsw.swmed.edu

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Isolation, Characterization, and Mapping of Human Islet Transcription Factors

M.A. Permutt[a], *H. Inoue*[a], *Y. Tanizawa*[a], *T. Tao*[a], *M. German*[b]

[a] Department of Internal Medicine, Division of Endocrinology, Diabetes, and Metabolism, Washington University School of Medicine, St. Louis, Mo. and
[b] Hormone Research Institute and Department of Medicine, University of California, San Francisco, Calif., USA

We have been testing the hypothesis that type 2 diabetes mellitus (T2DM) results from genetic defects in pancreatic islet β-cells, and that these inherited defects are responsible at least in part for failure to compensate for the insulin resistance of aging and obesity. To this end we have isolated a number of human islet homeodomain containing (homeobox) genes that are known to be key regulators of pancreatic islet development and insulin gene transcription. The first human gene isolated was insulin promoter factor 1 (IPF1/PDX1). The coding region was found to have two exons, with the deduced protein containing 283 amino acids. IPF1/PDX1 was mapped to chromosome 13q12(12.1) by fluorescent in situ hybridization analysis (FISH). A simple sequence repeat polymorphism (SSRP) was identified in an IPF1/PDX1 genomic clone, and this marker was used to incorporate the gene into the human linkage map at 20.9 cM on chromosome 13. The exon-intron boundaries were defined. These results led to the report of a patient homozygous for a nonsense mutation in this gene resulting in pancreatic agenesis, as well as maturity-onset diabetes of the young (MODY) in heterozygous relatives. Thus IPF1/PDX1 subsequently came to be known as the MODY 4 gene, although no other MODY families with mutations in this gene have been described. The role of IPF1/PDX1 in the pathogenesis of T2DM has been little studied.

CDX2/3 is an islet specific homeodomain protein identified in hamster insulinoma cells [1]. We isolated a human genomic clone, determined its genomic structure, found an SSRP and uniquely mapped the gene to the same region as IPF1/PDX1 [2]. Analysis in French families with T2DM was suggestive of linkage ($p = 0.01$) in lean (BMI < 27) patients [3] and further analysis is pending.

Islet-1 (Isl-1) is a unique LIM-homeodomain transcription factor that binds to the enhancer region of the insulin gene. A full-length human Isl-1 cDNA was isolated, and the genomic structure characterized. The cDNA encoded a predicted protein of 349 amino acids. By Northern and RT-PCR analysis, Isl-1 was most abundantly expressed in human islets, with a restricted pattern of expression in other adult human tissues. Analysis of genomic clones revealed that Isl-1 is encoded by 6 exons. The sequence of the proximal promoter region, including 426 bp upstream of the 5′-end of the cDNA, revealed potential regulatory elements. A search for variants in the gene and in the promoter by single-strand conformational polymorphism analysis (SSCP) in T2DM patients revealed 3 variants, none of which alter the predicted amino acid sequence. No variants were found in the promoter.

LMX1 is an LIM-homeodomain (LIM-HD) containing protein expressed selectively in insulin-producing β-cell lines. The human LMX1 (LMX1.1/LMX1A) gene was mapped by FISH to chromosome 1q22-q23. Subsequently, an LMX1-like exon, highly homologous to hamster LMX1, was found in exons trapped from chromosome 9 DNA. Using a probe to this LMX1-like chromosome 9 gene, we isolated a P1 genomic clone containing an LMX1-related gene, named LMX1.2/MX1B. An SSRP marker was identified in the P1 genomic clone that allowed us to genetically map the gene to chromosome 9. In addition, we refined the location of the human LMX1A gene to YAC clones that contained nearby SSRPs. Identification of this second LIM-HD-related gene may provide the opportunity to further understand the function of LIM-class homeobox genes, their roles in pancreatic islet development, and possible roles in the etiology of MODY and T2DM.

Nkx6.1 (gene symbol NKX6A), a new member of the NK homeobox gene family, was recently identified in rodent pancreatic islet β-cell lines. The pattern of expression suggested that this gene product might also be important for control of islet development and/or regulation of insulin biosynthesis. We cloned the human NKX6A gene. The predicted protein contained 367 amino acids and had 97% identity to the hamster protein. The highly conserved NK decapeptide and homeodomain regions were identical between human and hamster, suggesting functional importance of these domains. The coding region spanned approximately 4.8 kb and was comprised of 3 exons. The gene was localized to YAC clones and a nearby SSRP on chromosome 4 was identified. Using FISH, NKX6A was mapped to 4q21.2-q22.

The PAX4 gene encodes a transcription factor that has been shown to be essential for pancreatic islet β-cell development. We isolated a cDNA containing the full coding region and characterized its genomic organization. The cDNA encodes a predicted protein of 343 amino acids. Two DNA-binding motifs, a 123-amino acid paired domain and a 61-amino acid paired-type

homeodomain, exhibited high amino acid homology (73.2% and 62.3%, respectively) with the related domains of PAX6, a family member that is involved in differentiation of islet α-cells. Northern blot analysis of adult human tissues revealed expression predominantly in placenta and skeletal muscle, with low levels in pancreatic islets. Two SSRPs were identified at the PAX4 gene locus with heterozygosities of 0.50 and 0.83. Radiation hybrid analysis placed PAX4 and the newly identified markers on chromosome 7q31.3-32 border. The physical relationships between the markers were defined by mapping to a cosmid contig. To begin to assess the involvement of the PAX4 gene in susceptibility to T2DM, we performed affected sib-pair analysis by genotyping the two markers on Ashkenazi Jewish families with two or more affected members. This analysis revealed no evidence for linkage.

The isolation of these 7 human islet β-cell homeodomain genes, along with characterization of their genomic structures, identification of SSRP markers, and chromosomal mapping, will now provide the means to determine the possible role of mutations in these genes that contribute to the genetic risk for MODY and other forms of diabetes.

Insulin gene expression in adults is restricted to pancreatic islet β-cells. This limited pattern of expression, along with glucose-mediated regulation, has been shown to be determined at least in part by $\sim$350 base pairs of the insulin promoter, through cell transfection and transgenic models [4–7]. Two types of cis-acting elements within the insulin promoter have been proposed as contributors to its transcriptional control, the ubiqitous E-boxes with the consensus sequence GCCANNTG, and TAAT-boxes [8–10]. Two E-box motifs are conserved in the promoter regions of rodent and human insulin genes. These E-boxes are thought to be binding sites for basic helix-loop-helix (bHLH) transcription factors. The TAAT-motifs, more recently named the A-box [8], represent binding sites for homeodomain-containing proteins. Rat and human insulin promoter regions contain at least 3 of these A-boxes. Several islet homeodomain-containing transcriptional factors have been identified in rodents through binding to insulin promoter A-boxes, including isl-1 [11], lmx-1 [12], cdx-3 [12] and IPF1/PDX1/STF-1/IDX-1 [13–15]. Here we will review our strategies for isolation, characterization, and mapping of the human homologues of seven islet homeodomain (HD) genes.

Human LIM/homeodomain Gene Islet-1 (Isl-1)

The transcription factor Islet-1 (Isl-1), originally isolated from a hamster insulinoma (HIT) cell line, was shown to bind to nucleotide-247 to -198 of the rat insulin I gene [11, 16]. This gene was found to be a member of the LIM/homeodomain

family of proteins that are composed of 3 putative regulatory regions: two LIM domains (cysteine-histidine rich zinc-binding motifs) in the amino terminus of the protein, a homeobox domain near the middle, and a glutamine-rich transcriptional activation domain at the carboxyl end. Targeted disruption of this gene in mice results in failure of normal islet β-cell development and neonatal diabetes [17].

To isolate the human Isl-1 gene, a partial Isl-1 cDNA was synthesized by reverse transcription-polymerase chain reaction (RT-PCR) from human islet RNA using primers designed from the rat sequence [18, 19]. Human islet cDNA libraries were screened and one cDNA that was 150 bp larger than the published rat Isl-1 sequence was obtained. This cDNA contained 2,395 bp plus additional poly(A) residues that contained an open reading frame encoding a predicted protein of 349 amino acids, molecular weight 39 kD (Genbank accession #UO7559). In addition, an unusual finding was that one-third (8/24) of the cDNAs isolated appeared to be incompletely processed Isl-1 transcripts.

Expression of Isl-1 in human tissues was found to be predominantly in islets in adults, although neural tissue was not tested. By RT-PCR analysis, after extensive amplification (45 cycles), Isl-1 mRNA was not detected in spleen, liver, or pancreas, confirming restricted expression of the gene [20, 21]. Southern blot analysis revealed that the gene was single copy in the genome. The cDNA was used to isolate genomic clones in the bacteriophage λ. The gene was encoded by 6 exons. Exons 2 and 3 each encode an entire LIM domain. The homeodomain is completely contained within exon 4. Sequence of 426 bp of genomic DNA upstream of the 5′-end of the Isl-1 cDNA revealed no TATA box, but other regulatory motifs were observed [19, 20].

A highly polymorphic SSRP was found in the Isl-1 gene with heterozygosities of 0.69–0.87 in 3 racial groups, and linkage analysis in CEPH pedigrees uniquely placed Isl-1 on chromosome 5q [18]. The SSRP at the Isl-1 locus was employed to evaluate mutations in this gene as a possible contributor to the pathogenesis of NIDDM. Allelic frequencies did not differ between patients with T2DM (n = 165) and nondiabetic controls (n = 163) in St. Louis African-Americans and Nigerians. Linkage analyses in 15 nonglucokinase MODY pedigrees indicated that linkage could be rejected (LOD score < -3.0) over a distance of 15 cM. The probands of 75 French Caucasian T2DM pedigrees were screened by PCR-SSCP, and 3 variants were identified, none of which affect the predicted amino acid sequence of Isl-1 [19].

Human Insulin Promoter Factor 1 (IPF1/PDX1) Gene

IPF1/PDX1 was cloned as a homeodomain-containing protein that is selectively expressed in adult islet β-cells [22]. This protein also appears to

transactivate insulin promoter activity by interaction with the so-called A1 region of the rat insulin I gene. In embryonic development, IPF1/PDX1 expression is initiated in islets prior to hormone gene expression, and is restricted to the dorsal and ventral walls of the primitive foregut where pancreas will later form [14]. IPF1/PDX1-deficient mice, created by gene targeting, selectively lack a pancreas at birth [23]. These data suggest that IPF1/PDX1 is a key regulator of pancreatic islet development and insulin gene transcription in β-cells.

To isolate the human IPF1/PDX1 gene, a set of primers complementary to a part of the homeodomain of the mouse IPF1/PDX1 gene were synthesized and a fragment of human genomic DNA obtained [2]. A P1 clone was isolated, and the entire coding region of the gene was contained within two exons over a 5 kb region of genomic DNA. The coding region showed 83% nucleotide identity with the mouse IPF1/PDX1 gene, and 88% amino acid identity. Southern analysis revealed that the gene was a single copy in genomic DNA.

The chromosomal localization of IPF1/PDX1 was determined with DNA from a panel of human-rodent hybrid cell lines and by FISH analysis to be on 13q12(12.1). An SSRP for linkage mapping was found (GenBank Accession: U40404(with heterozygosity = 0.32. We also identified a highly polymorphic SSRP marker, cdx3GA1, in the human CDX2/3 genomic λ clone (GenBank Accession No. U46755) and two-point linkage analysis inferred that the most likely location for IPF1/PDX1 was at $\theta = 0$ from the cdx3GA1 locus. To search the homeodomain region for potential variants in T2DM, SSCP analysis was performed in 61 Japanese patients and no variants were found.

Human CDX2/3 Gene

CDX3, a member of the caudal-type homeobox family of genes, was cloned from a HIT cell cDNA library and shown to transactivate the insulin gene [1]. Mouse CDX2 is important in tissue specific expression of intestinal genes, and hamster CDX3 and mouse CDX2 were shown to be the same protein. Targeted disruption results in fetal lethality, while heterozygotes have growth retardation and adenomatous intestinal polyps [24].

Using the hamster cDNA as probe, we isolated a human P1 clone that encoded CDX2/3 and it was found to have 93% amino acid identity to the mouse gene (Y. Tanizawa et al., unpublished observations). Three exons were found, and intron/exon boundaries defined. Analysis in French families indicated linkage ($p = 0.01$) in lean (BMI < 27) T2DM patients [3], and further analysis is pending.

Human LMX1A and LMX1B Gene

LMX1 is another member of the LIM-homeodomain (LIM-HD) class of proteins, characterized by the presence of two tandem cysteine/histidine-rich, zinc-binding LIM domains, known as LIM1 and LIM2, respectively [12]. LMX1 was originally cloned from a HIT cell cDNA library, and shown to transactivate the insulin gene, along with the bHLH protein E47/Pan-1 [25]. Several LIM-HD proteins have been implicated in the control of differentiation of specific cell types [26], although the functional role of the LMX1 gene in the regulation of islet β-cell differentiation has not been established.

To obtain the human LMX1 gene, a search of the human dbEST database with the hamster LMX1 cDNA sequence disclosed two highly homologous sequences (GenBank Accession No. T12579 and T12628) [27]. Interestingly, these LMX1-related ESTs were originally identified by exon trapping of human chromosome 9 [28]. This finding was inconsistent with reported FISH analysis of the LMX1 gene placing it on chromosome 1 [29]. These discrepant results suggested that the chromosome 9 LMX1-related ESTs may represent a closely related but distinct gene(s).

Based on the chromosome 9 LMX1-related sequences, primers were synthesized for RT-PCR analysis of RNA from human pancreatic islets. A 482 bp product was obtained (LMX1B, GenBank Accession No. U77457) and compared with hamster LMX1. The human fragment contained the entire LIM2 domain, the homeodomain, and a small part of the polyglutamine-rich 3′-transcriptional activator region. Sequence identity between the 482 bp RT-PCR product and the corresponding hamster LMX1.1 sequence was 73% and 92% at the nucleotide and amino acid levels, respectively. The DNA-binding homeodomains were 100% identical at the amino acid level. Concomitant with this analysis of the human LMX1-related gene, using the same human chromosome 9 exon-trapped EST sequence, the HIT cell cDNA library was rescreened and the hamster LMX1.2 cDNA was cloned (MS German, submitted). In addition, the chicken C-LMX1 gene was also recently cloned from limb and spinal cord cDNA libraries using the hamster LMX1 sequence [30, 31], and it was noted to contain 70% amino acid identity to hamster LMX1. Comparison of these sequences revealed highest homology among our human cDNA (LMX1B), chicken C-LMX1, and hamster LMX1.2. The results suggested that these sequences represent homologues of the same gene and that, although highly-related to the hamster LMX1 gene, they are different. We then named our human LMX1-related gene as human LMX1.2/LMX1B to disinguish it from hamster LMX1, now called LMX1.1/LMX1A. Hamster LMX1.1/LMX1A has been shown to have restricted tissue distribution, predominantly in hamster insulinoma (HIT T15) cells [12]. LMX1.2/

LMX1B expression was detected in most of the tissues examined, and particularly in the testis, thyroid, duodenum, skeletal muscle, and pancreatic islets.

Sequence tagged sites (STSs) for LMX1A and LMX1B were designed. We screened a CEPH 'B' yeast artificial chromosome (YAC) library (Research Genetics, Inc. Huntsville, Ala.), and no informative YAC clone was obtained using LMX1B primers. For the human LMX1A, several YAC clones were identified that contained highly polymorphic markers that genetically mapped at 204 and 206 cM on chromosome 1, confirming the previous FISH study [29]. In addition, we identified an SSRP marker (hLMX1.2CA1, GenBank Accession No. U77458) in a P1 genomic clone containing the LMX1B gene, and this marker was uniquely placed on chromosome 9 between markers that were genetically mapped at 141 and 146 cM. Several linkage markers nearby have been cytologically mapped suggesting 9q32-34.1 as the cytological location of LMX1.2.

It is interesting that two such highly related LIM-HD proteins are co-expressed in pancreatic islet β-cells. Although the functional significance of this observation remains to be determined, it has been shown that, similar to the LMX1A protein, LMX1B can also activate transcription of the insulin gene in combination with the bHLH protein E47/Pan-1 (MS German, submitted).

Subsequent to our report of the existence of this new LMX gene (LMX1.2/LMX1B) mapping to chromosome 9, geneticists positionally cloning the Nail-Patella Syndrome (NPS) gene had narrowed the critical region in families to 9q32. They chose LMX1B as a candidate for NPS [32, 33]. Based on the sequence of the hamster and our partial LMX1B sequence, they characterized a human genomic clone and defined at least 8 exons. Disease-causing mutations were identified in a number of families that cosegregated with the disease. No studies of LMX1A or LMX1B have yet been reported in diabetes.

Human Nkx6.1 (NKX6A) Gene

Nkx6.1 (gene symbol NKX6A), a distantly related member of the NK homeobox gene family, was recently identified in rodent islet and insulinoma cell lines by PCR amplification with a primer designed from a conserved region of the hamster cdx3 gene [12]. Although the function of NKX6A is unknown, its expression pattern was predominantly in islet β-cells. We therefore isolated the human gene encoding NKX6A, characterized its genomic structure, and determined its chromosomal assignment screening a yeast artificial chromosome (YAC) library and FISH analysis [34].

A P1 human genomic clone was isolated using a set of primers selected from the partial sequence (251 bp) of a λ clone containing the homeodomain region of the human NKX6A (M. German, unpublished data). The coding sequence of the last exon containing the homeodomain and a stop codon was obtained. This sequence was highly homologous to the hamster sequence (92% at the nucleotide level and 97% at the amino acid level). Next because no further information on the genomic structure, intron size or human sequence of the N-terminal region was known, a PCR strategy was devised assuming high nucleotide similarity between the N-terminal regions of the hamster and human genes. Long PCR was performed on P1 DNA and PCR products were directly sequenced and compared to the hamster sequence. Based on newly obtained human sequence, P1 sequencing was continued until the entire coding sequence and exon-intron boundaries were obtained.

The coding region of NKX6A was approximately 4.8 kb in size and comprised 3 exons. The predicted protein sequence of the human NKX6A included 367 amino acids (GenBank Accession U66797, U66798, U66799), 1 amino acid (3 bases) larger than the hamster Nkx6A protein, and had 97% overall identity to the hamster sequence. The NK decapeptide and homeodomain regions were 100% identical between the hamster and human genes, suggesting functional importance of these domains. The 5′-end of exon I and the 3′-end of exon III were not mapped, and thus additional exons may exist.

NKX6A primers were also used to screen the CEPH 'B' YAC library (Research Genetics, Inc. Huntsville, Ala.), and positive YAC clones were found. D4S1538, a polymorphic marker on the YACs, was genetically mapped at 96 cM on chromosome 4 on the Genethon map [35]. NKX6A was localized to 4q21.2-q22 by FISH analysis.

Human PAX4 Gene

PAX genes are members of a family of developmental control genes encoding nuclear factors that play critical roles during fetal development [36]. These genes have sequence homology to the *Drosophila* segmentation genes, and nine have been described in mouse. Based on a highly conserved 128aa paired domain at the amino terminus, an octapeptide, and a paired-box type homeodomain, these nine genes have been separated into four groups, PAX4 and PAX6 are closely related members of Group IV [37]. Results of recent gene targeting experiments revealed that Pax4 and Pax6 are involved in pancreatic islet development. Pax4 mutant mice lacked β-cells and δ-cells [38], whereas Pax6 mutant mice lacked α-cells [39, 40].

Prior to our studies, the only human PAX gene not isolated was PAX4, and initially only a partial mouse Pax4 sequence was known. A BLAST search of public databases with the partial sequence of the mouse Pax4 cDNA revealed highly homologous sequences in a human cosmid clone that had been mapped to chromosome 7. We found that this human homologue appeared to be present in five separate fragments within a 2.5 kb region of this cosmid clone. Based on the sequence of this human homologue, we designed oligonucleotide primers and a partial cDNA of human PAX4 was amplified by PCR from human placenta cDNA [41]. 5′ and 3′ RACE (rapid amplification cDNA ends) reactions were performed, and we isolated cDNA containing the complete coding region of the human PAX4.

Interestingly, the isolated human PAX4 cDNA had relatively low homology to mouse Pax4 (76.4% at the amino acid level), compared to that between human and mouse for other members of the PAX gene family ($>95\%$ at amino acid level). These results originally suggested that our isolated cDNA was not PAX4, but rather a new PAX gene. To examine this hypothesis, human genomic DNA was subjected to Southern blot analyses with fragments of the human placental PAX4 cDNA and the mouse Pax4 cDNA as probes. The results of these studies indicated that the isolated cDNA represented the product of the human PAX4 gene as a single copy in the human genome. Examination of the mouse and human sequences revealed $>90\%$ identity within the paired- and homeodomains respectively, while the carboxyl terminus differed considerably, suggesting alternative splicing of the mRNAs.

The number and size of exons and the intron/exon boundaries were defined by comparison of the cDNA with the entire sequence of the genomic chromosome cosmid g1572c264. Interestingly PAX4 was found to contain 9 exons in contrast to the human PAX6 gene containing at least 13–14 exons. The 5′-untranslated region of PAX6 in encoded by 3 exons, suggesting that further 5′-exons may be identified in human PAX4. The expression of mRNA for the PAX4 gene in normal adult human tissues was determined by Northern analysis of poly(A)$^+$ RNA and shown to be predominantly in placenta and skeletal muscle. To confirm the expression of PAX4 mRNA in pancreatic islets, RT-PCR was performed, revealing low levels of expression, and without alternatively spliced isoforms of PAX4 mRNA between placenta and pancreatic islets.

Two SSRPs were identified (GenBank AF047018 and AF047019) within 16 kilobases of the PAX4 gene. To further refine the location of this gene on the genetic and physical maps of chromosome 7q, these markers were used to screen the Stanford Radiation Hybrid G3 panel (Research Genetics, Inc.). The PAX4 gene was mapped to human chromosome 7q31.1-32 border.

To assess the possible implication of the PAX4 gene in diabetes, we performed affected sib-pair analysis by genotyping the new markers. Eighty-five

Table 1. Human islet homeobox genes

Gene	Mutations Mouse/human		Mapping		Exon #	Prom.
	Natural	Targeted	FISH	Linkage/phys		
ISL1	–/–	Yes	nd	5q(CA)$_n$ ~70 cM	6	426 bp
PDX1/IPF1/P	–/+	Yes	13q	(CA)$_n$ ~31 cM	2	283 bp
CDX2/3	–/+	Yes	13q	(CA)$_n$ ~31 cM	3	56 bp
LMX1A	–/–	No	1q22-q23	~205 cM YAC	nd	nd
LMX1B	–/+	No	9q32-34.1	~146 cM YAC	8	nd
NKX2.2	–/–	Yes	20p11	~30 cM YAC	2	90 bp
NKX6A	–/–	Unpub.	4q21.2-q22	96 cM YAC	3	nd
PAX6	+/+	Yes	11p13	32 cM YAC	13+	>2.4 Kb
PAX4	–/–	Yes	7q31.1-32	134 cM RH	9	nd

nd = Not determined; phys. = physical mapping to either YACs (yeast artificial chromosomes) or by radiation hybrid (RH) analysis; prom. = putative promoter region.

Ashkenazi Jewish families with at least two siblings with overt T2DM were examined, and found to have no evidence for excess allele sharing. Our results indicate that genetic variation in the human PAX4 gene region is unlikely to be a major contributor to the pathogenesis of T2DM in this group. These results do not exclude the possibility of a minor role for the PAX4 gene in a polygenic model however. It is also possible that PAX4 may contribute to T2DM in other racial/ethnic groups, and the markers defined here will be useful for these analyses.

Summary and Conclusions

We now have isolated, characterized and mapped 7 human islet homeodomain genes. The general fetures of these genes, along with those of a recently characterized new member of the NK family, NKX2.2, also expressed in islets [42] and PAX6 [39] are shown in table 1. Among these islet homeodomain genes natural mutations have been observed in mouse only for Pax6, small eye mutation [43]. In humans, natural mutations have been observed in IPF1/PDX1 [44], LMX1B [32, 33], and PAX6 (aniridia and WAGR [45]). Interestingly targeted disruption of islet homeodomain genes in mice have been accomplished for each gene for which the genomic structure has been deter-

Table 2. Islet homeobox genes and diabetes

Gene	Linkage		Mutations/variants	
	MODY	T2DM	MODY	T2DM
ISL1	Neg	Neg-unpub.	nd	French Cauc.
PDX1/IPF1	1 family	Neg-unpub.	MODY/Early-onset T2DM in Japanese (G. Bell, unpub.)	
CDX2/3	Nd	Neg-unpub.	nd	nd
LMX1A	Nd	nd	nd	nd
LMX1B	Nd	Neg-unpub.	nd	nd
NKX2.2	Nd	nd	MODY/Early-onset T2DM in Japanese (G. Bell)	
NKX6A	Nd	Neg-unpub.	nd	nd
PAX6	Nd	nd	nd	nd
PAX4	Nd	Neg	nd	nd

For references see text.

mined. All the listed genes have been mapped, initially by linkage analysis in CEPH pedigrees with SSRP markers, but more recently by mapping of STSs to YACs or by radiation hybrid analysis. The genomic organization for most have been characterized, along with intron/exon borders, so that the genes can be amplified from genomic DNA and subjected to mutation detection via either SSCP or dHPLC [46], or directly by sequencing. The promoter regions have in many cases been defined so that variants could be sought in putative regulatory regions.

The potential roles of these imporant islet developmental regulatory genes in many cases known to be required for insulin and other islet gene expression in the insulin deficiency of T2DM have been little studied. Our lab has conducted preliminary linkage analyses on 100 Ashkenazi Jewish affected sib pairs for the indicated genes in table 2. No significant linkage was observed. This should not be surprising for a polygenic disease. Linkage in MODY families has only been reported for ISL1 [18]. Mutation analysis in MODY has been reported for IPF1/PDX1 and NKX2.2 in Japanese, and no variants accounting for the disease were detected [42]. For T2DM only the ISL1 gene has been screened for mutations in French Caucasians [3]. As can be seen in table 2, the potential

roles of these genes in MODY and T2DM have been little assessed. Promoter mutations resulting in decreased expression and haplo-insufficiency should also be considered.

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M.A. Permutt, MD, Washington University School of Medicine,
Division of Endocrinology, Diabetes, and Metabolism,
660 South Euclid, Campus Box 8127, St. Louis, MO 63110 (USA)
Tel. +1 314 362 8680, Fax +1 314 747–1309, E-Mail apermutt@imgate.wustl.edu

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The Glucokinase System and the Regulation of Blood Sugar

Franz M. Matschinsky[a], *Elizabeth Davis*[a], *Antonio Cuesta-Muñoz*[a], *Carol Buettger*[a], *Ian Sweet*[a], *Michael Moates*[b], *Mark A. Magnuson*[b]

[a] Department of Biochemistry and Biophysics and the Diabetes Research Center, University of Pennsylvania School of Medicine, Philadelphia, Pa. and
[b] Department of Molecular Physiology and Biophysics, Vanderbilt University Medical School, Nashville, Tenn., USA

The pancreatic β-cell threshold for glucose stimulated insulin release (GSIR) and the hepatic glucose usage and/or production are the critical determinants of maintaining interprandial blood glucose levels at about 5 m*M* in individuals with normal insulin sensitivity [1, 2]. The glucokinase (GK) system with its complementary roles in β-cells (GKB) and hepatocytes (GKL) is critically involved in maintaining the setpoint of the hepato-pancreatic feed back loop at 5 m*M* glucose [1, 3] (fig. 1). We hypothesize that the GKB (the β-cell glucose sensor) plays the predominant role of this two component system because under physiological conditions it regulates the glucose threshold for GSIR, whereas GKL plays an auxiliary role because it seems to lack a hepatic glucose threshold for intermediary metabolism and because of the impact of G6Pase which codetermines glucose net usage. The β-cell threshold of 5 mM for GSIR is governed primarily by the adenine nucleotide sensitive potassium channel and the voltage sensitive calcium channel and is reached when the β-cell glucose usage (determined by GKB catalyzed glucose phosphorylation) achieves about 25% of its theoretical maximum capacity with the substrate glucose and ATP at saturation levels as will be explained in the report that follows. Any change of GKB activity causes inversely related shifts of the threshold for GSIR [4, 5]. Changes need not be large (i.e. 25%) to be biologically significant.

A minimal mathematical model defining the GKB dependency of the β-cell glucose threshold has been formulated and accurately predicts the basal

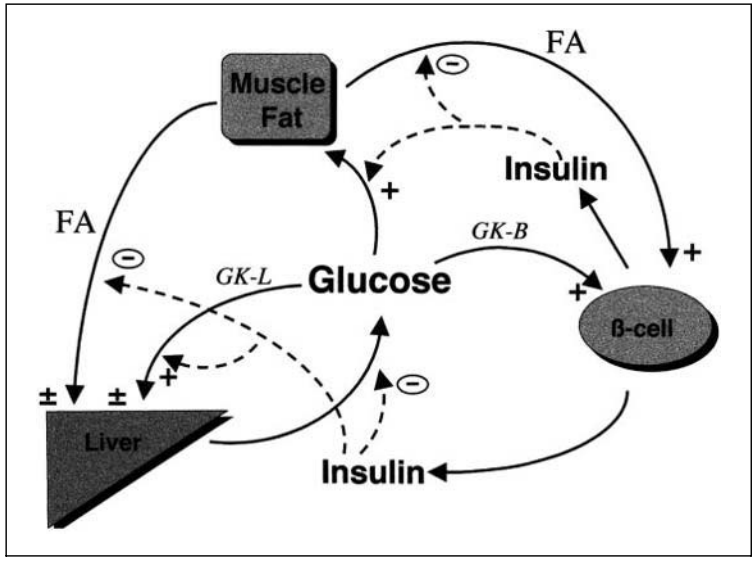

Fig. 1. Central role of the glucokinase system in blood sugar control. The figure is for the most part self-explanatory. Glucose and FA are taken up by the liver and may modify metabolic pathways negatively or positively. FA appear to enhance gluconeogenesis but may inhibit liponeogenesis. Glucose may enhance glycogen synthesis but may inhibit urea production. This is indicated by (±) signs.

or interprandial blood sugar level which can be presumed to be equivalent to the threshold for GSIR [4, 5]. Equations 1–3 describe this relationship:

$$\beta\text{-GPR} = \frac{2\text{GKB} \times k_{cat} G^h}{Gb + {}^{G}S_{0.5}^h} \times \frac{ATP}{ATP + {}^{ATP}K_m} \tag{1}$$

$$\frac{\beta\text{-GPR}}{2\text{GKB} \times k_{cat}} = \frac{G^h}{G^h + {}^{G}S_{0.5}^h} \times \frac{ATP}{ATP + {}^{ATP}K_m} \tag{2}$$

$$\frac{\beta\text{-GPR}}{2\text{GKB} \times k_{cat}} = \frac{5^{1.8}}{5^{1.8} + 8.4^{1.8}} \times \frac{2.5}{2.5 + 0.34} = 0.25 \tag{3}$$

βGPR is the glucose phosphorylation rate of the β-cell expressed in terms of mole of glucose used per gram of β-cell tissue per second; GKB refers to the β-cell GK content (mol/g tissue) where GKB is a constant; k_{cat} is the GK turnover number or rate constant when saturated with both substrates (sec^{-1}); G is the blood sugar or intracellular β-cell glucose level (m*M*), *h* is the GK Hill coefficient for the substrate glucose; ${}^{G}S_{0.5}$ is the glucose level at half maximal activity of GK (m*M*); ATP is the β-cell level of the second substrate (m*M*); ${}^{ATP}K_m$ is the affinity constant for ATP(m*M*). Equations 2 and 3 are

unitless. At $G = 5\,mM$ and $ATP = 2.5\,mM$ β-cell glucose usage reaches the threshold rate of about 25% of normal capacity ($2GKB \times k_{cat} = 100\%$). Any change of the kinetics or the expression of β-cell GK alters the threshold according to equation 4

$$0.25 = \frac{\beta\text{-GPR}}{2GKB \times k_{cat}} = \frac{G^{h\delta}\alpha\beta}{G^{h} + {}^{G}S_{0.5}^{h\delta}\gamma} \times \frac{ATP}{ATP + {}^{ATP}K_{m}\varepsilon} \tag{4}$$

where α, β, γ, δ and ε are factors that modify GK by changing the enzyme's rate constant (α) its expression (β), its affinity for glucose (γ), its cooperativity with the substrate glucose (δ), or its affinity for ATP (ε). The variable in this equation is glucose, the threshold for GSIR.

The glucose threshold of the β-cell may be plotted as a function of the normalized β-cell GK activity index Ia (equation 5).

$$Ia = \frac{k_{cat}}{{}^{G}S_{0.5}^{h}} \times \frac{ATP}{ATP + {}^{ATP}K_{m}}. \tag{5}$$

This index (expressed in terms of $1/sec \times mM^{h}$) accounts for practically all factors that determine the enzymatic function of β-cell GK and relates β-cell GK to the threshold for GSIR (fig. 2). It is unitless when normalized.

The autosomally dominant inherited GK-DM (usually called MODY2) and GK-HI syndromes offer a natural opportunity unmatched by experimental models including GK knockout mice to test these results of mathematical modeling [6, 7]. Since β-cell GK expression is determined by gene dosage the influence of both alleles on β-cell GK content and characteristics needs to be considered. Assuming a heterozygous genotype with one normal (w) and one mutant (m) gene the equation (2) may be reformulated.

$$\frac{\beta\text{-GPR}}{2GKB^{w} \times {}^{w}k_{cat}} = \frac{0.5G^{hw}}{G^{hw} + {}^{wG}S_{0.5}^{hw}} \times \frac{ATP}{ATP + {}^{wATP}K_{m}} + \frac{0.5G^{hm}}{G^{hm} + {}^{mG}S_{0.5}^{hm}} \times \frac{ATP}{ATP + {}^{mATP}K_{m}} \times \frac{{}^{m}k_{cat}}{{}^{w}k_{cat}} \times \frac{GKB^{m}}{GKB^{w}} \tag{6}$$

where ${}^{m}k_{cat}/{}^{w}k_{cat}$ and GKB^{m}/GKB^{w} are factors that indicate an alteration of the rate constant and expression of the mutant enzyme, respectively. The threshold concentration of glucose for stimulation of secretion may then be calculated using reasonable assumptions and the results may be expressed in form of a GK-Ia/glucose threshold diagram using the normalized β-cell GK activity index Ia (equation 7).

$$Ia = \frac{\frac{{}^{w}k_{cat}}{{}^{wG}S_{0.5}^{hw}} \times \frac{ATP}{ATP + {}^{wATP}K_{m}} + \frac{{}^{m}k_{cat}}{{}^{mG}S_{0.5}^{hm}} \times \frac{ATP}{ATP + {}^{mATP}K_{m}}}{2}. \tag{7}$$

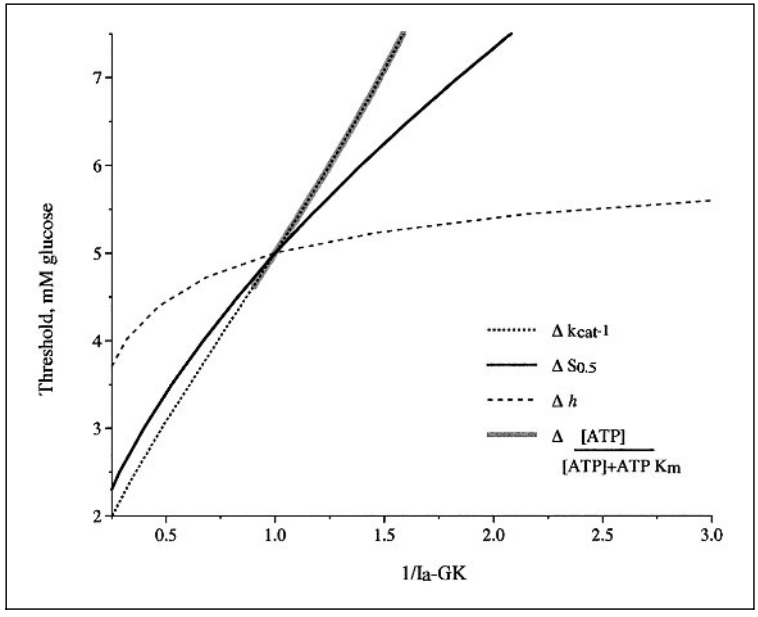

Fig. 2. Impact of individual parameters of the mutant GK activity index Ia on the β-cell threshold for glucose-stimulated insulin release. Only that part of expression (7) that refers to the mutant allele is used rather than the lumped constant Ia. The mutant GK index is normalized, a value of 1 referring to the physiological threshold of 5 m*M* computed with the kinetic constants of wild-type GK and using a β-cell ATP level of 2.5 m*M*. The figure indicates that the control strengths of k_{cat} and glucose $S_{0.5}$ are comparable in the physiological glucose range of 3 to 7 m*M*. ATP affinity influences the system only when the ATP K_m increases above the normal level of 0.31 m*M* and then the effects overlaps with that of equivalent k_{cat} changes. The impact of the Hill coefficient is small and is apparent only when it decreases.

This lumped GK activity index accounts for the characteristics of both alleles of β-cell GK.

A representative database on the kinetics of recombinant wildtype GK, mutant GK from GK-DM and mutant GK from GK-HI syndromes (unpublished data) together with the corresponding clinical data from an extended literature [6] may then be used to test the model (tables 1, 2). The database was obtained from kinetic and thermolability studies of human recombinant wildtype GK, one incidental GK mutant (D158A) previously used as a control enzyme by others [8, 9] and 19 spontaneous mutants causing MODY or hyperinsulinemia linked to GK. A wide range of kinetic changes were found in 15/19 spontaneous mutants. The $^{G}S_{0.5}$, the k_{cat}, the Hill number and ATP

Table 1. GST-GK kinetics database. The mutant data are expressed in relative terms compared to wild-type enzyme. k^1_{cat} and k^2_{cat} refer to the results with two different assays. Since k^2_{cat} is less influenced by enzyme instability and corrects for changes of ATP affinity it was used for all modeling studies. D158A was included in this study because it was previously used by others as control enzyme before this incidental mutation was discovered. All data are corrected for a temperature of 37 ºC

Mutant	Yield	$S_{0.5}$	h	I.P.	ATP K_m	k^1_{cat}	k^2_{cat}	Mutant
WT*	**15.94 mg/l**	**8.33 m*M***	**1.80**	**4.12 m*M***	**0.31 m*M***	**51.54 s^{-1}**	**61.73 s^{-1}**	**WT***
A53S**	1.16	0.97	1.00	0.99	0.71	0.88	0.77	A53S**
E70K	1.65	1.38	0.99	1.37	1.16	0.84	0.56	E70K
G80A	1.18	115.85	0.36	n.a.	24.87	0.00	0.00	G80A
H137R	0.81	0.90	1.00	0.91	0.77	0.87	0.73	H137R
D158A***	1.55	0.44	0.94	0.40	1.06	1.01	0.99	D158A***
T168P	1.19	19.22	0.51	n.a.	30.10	0.01	0.02	T168P
G175R	1.66	2.35	0.97	2.24	1.45	0.33	0.37	G175R
V182M	1.76	4.19	0.94	3.81	0.35	0.24	0.20	V182M
V203A	1.66	7.01	0.85	5.07	2.84	0.24	0.31	V203A
M210T	0.45	28.21	0.76	11.75	18.32	0.12	0.43	M210T
C213R	0.66	7.01	0.98	6.77	2.74	0.27	1.14	C213R
V226M	1.19	2.15	0.78	1.23	18.52	0.83	1.00	V226M
T228M	1.36	n.a.	n.a.	n.a.	n.a.	n.d.	n.d.	T228M
G261R	0.13	14.95	1.17	17.84	12.35	0.02	0.31	G261R
E300K	0.44	1.24	1.03	1.30	1.48	0.66	0.96	E300K
L309P	0.02	1.14	1.07	1.24	1.87	0.02	0.05	L309P
S336L	1.55	0.65	0.63	0.13	70.00	0.02	0.09	S336L
V367M	1.45	0.91	1.00	0.92	1.00	1.13	1.06	V367M
K414E	1.09	1.48	0.93	1.35	5.58	0.21	0.76	K414E
V455M	0.52	0.38	0.92	0.33	0.81	0.94	0.97	V455M

* Mean of nine preparations.
** Relative to wild-type value which is considered 1.00, and is therefore unitless.
*** Incidental mutant.

K_m were affected, usually in combination. Exceptions were D158A and V455M which showed singular $^GS_{0.5}$ changes from 8.4 to 3.2 and 3.4 m*M*, respectively, which fully explains the hyperinsulinemia in V455M [7] but also shows that D158A is not an acceptable control enzyme [5, 8]. Four of the mutants were kinetically indistinguishable from wild-type (A53S, H137R, E300K and V367M) but three of these were thermally more labile than the wildtype. E300K was investigated most thoroughly including in vitro thermolability assays of recombinant GST-E300K-GKB and cell biological assessment of enzyme expression and stability using viral vector technology and the β-HC9

Table 2. Clinical database and model predictions for basal blood glucose in MODY2 and GK-HI [6, 7, 21–23]. Instability mutants H137R, E300K and V367M were considered reduced to negligible levels, i.e. $GKB^m \leq 0.1$

	Fasting blood glucose, m*M*		
	Controls	MODY2 mutations	HI-GH mutations
Mathematical model	5 (by definition)	$6.7 \pm 0.1^{\#}$ (predicted)	3.09 (predicted)
Velho et al. [6]	5.0 ± 0.5* (n = 341)	7.0 ± 1.1* (n = 260)	
Page et al. [21]	4.9 ± 0.4* (n = 12)	7.3 ± 1.8* (n = 17)	
Byrne et al. [22]	$5.0 \pm 0.02^{\#}$ (n = 6)	$6.7 \pm 0.1^{\#}$ (n = 6)	
Shimada et al. [23]	$5.1 \pm 0.2^{\#}$ (n = 8)	$6.8 + 0.4^{\#}$ (n = 6)	
Glaser et al. [7]			2.25 (n = 2)

* Means ± SD; # means ± SEM.

for expressing the E300K [unpubl. data]. This battery of tests showed that E300K is indeed an instability mutant with virtually normal kinetics. One of the mutants studied so far was entirely normal and may represent a polymorphism (A53S). Using this database the β-cell glucose thresholds or interprandial blood glucose levels were calculated as a function of the respective GK Ia (fig. 3). It may be extrapolated that the expression of thermal lability mutants is reduced in the β-cells, e.g. GKB^m/GKB^w may be as low as 0.1 or less and the Ia can be corrected by this factor. It was then found that 17/18 MODY2 mutants have apparent thresholds between 7 and 10 m*M* glucose with the majority approaching 10 m*M* (fig. 3). This value is significantly higher than observed clinically in a large population of MODY2 patients (table 2). However there is good evidence that high glucose induces β-cell GK by a post-translational mechanism resulting in partial restitution of its activity, certainly of the component controlled by the wildtype allele [10]. By making this adjustment with an experimentally derived factor of ±0.2/m*M* blood glucose change [10] and a successive approximation protocol to estimate the adaptation, the modeled data from 17 MODY2 mutants predict an average interprandial blood sugar of about 6.7 m*M*, very close to 7.0 m*M* found in a large group of MODY2 patients (table 2). It is remarkable that the clinical picture differs little in these patients [6] even though the severity of the kinetic or expression change of the mutant GKB covers a very wide range of about 1,000-fold in terms of the Ia. It may be methodologically difficult to ascertain GK mutants which are so mild that they result in interprandial blood sugar levels that are significantly higher than 5 m*M* but lower than 7 m*M* which may explain the homogeneity of clinical cases.

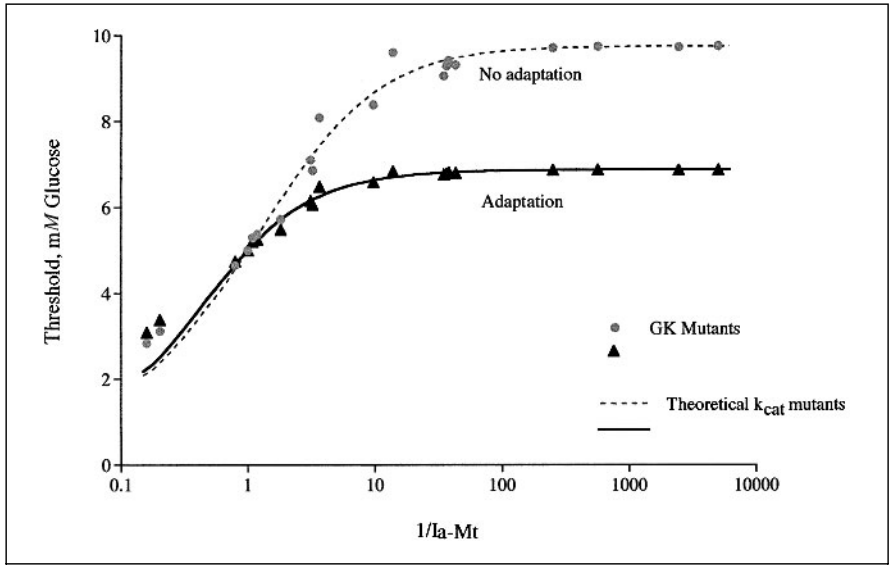

Fig. 3. Effect of GK mutants on the β-cell threshold for GSIR. The GK kinetics data base of table 1 and equation (6) were used to calculate the glucose threshold for insulin release for wild-type and mutant enzymes. Both the adapted and nonadapted situations are shown. The broken or continuous lines represent theoretical curves and were obtained by systematic changes of the K_{cat} only. The GK mutant data represented by solid circles or triangles were plotted as a function of the mutant GK activity index accounting only for the mutant allele rather than the lumped GK index which would account for both alleles. Note that the three instability mutants cluster around the wild-type, since instability was not considered in this graph. Accounting for instability would change the 1/Ia-Mt from between 100 and 10,000 fold depending on the change of GK expression.

The mathematical model emphasizes basal insulin secretion and the glucose threshold for GSIR. Basal or interprandial insulin release is critical for glucose homeostasis contributing an estimated 50% to total insulin released daily from the pancreas assuming three mixed meals. It probably results from the action of non glucose fuels, most importantly amino acids and fatty acids [11] with glucose playing an essential permissive and thus GK dependent role. It is further speculated that ATP serves as metabolic coupling factor just as proposed for GSIR but that transmission does not require depolarization of the cell membrane nor an increase of intracellular Ca^{2+} which contrasts with GSIR. ATP may affect exocytosis directly [12]. It is extrapolated that the results are also indicative of the β-cell response to hyperglycemia. Postprandial elevation of blood sugar from 5 m*M* to the GK $S_{0.5}$ of 8.4 m*M* increases β-cell glucose phosphorylation to 50% of its capacity and GSIR as much as

five-fold [13]. If the present modeling approach is extended to assess the effect of GK-DM and GK-HI mutants on β-cell glucose responsiveness at this critical blood glucose level equivalent to the $S_{0.5}$ of GK, the findings closely resemble those obtained for the threshold of GSIR provided adaptation of wildtype GK to hyperglycemia is incorporated in the model (data not shown).

These considerations show that the minimal model as described here accurately predicts the clinical picture in GK-DM and GK-HI patients and illustrates again and very poignantly the crucial role of the β-cell GK glucose sensor system in the regulation of blood sugar. Relatively small alterations of GK activity have biologically significant effects on basal blood sugar, the glucose threshold for GSIR and on physiological GSIR attesting to the validity of the concept. These conclusions also identify β-cell GK as a very sensitive target for manipulations by hormones and transmitters, by macro- and micronutrients and clearly point to this enzyme as a potential drug target. Much new insight can be expected to come from research programs guided by such considerations.

The β-cell may gradually lose its predominant position in blood sugar regulation in severe insulin resistance states for example in obesity [14, 15] and in late pregnancy [16]. Using isolated islet preparations from experimental animals with these conditions the β-cell glucose threshold is usually found to be lower than the interprandial blood glucose level implying other processes are probably gaining increasing influence. This β-cell threshold shift, whatever its molecular basis, seems to explain in large part the interprandial hyperinsulinemia usually found in such conditions. Hyperplasia of the β-cells may also contribute to this basal hyperinsulinemia. The physiological postabsorptive off-switch of β-cells appears to be less effective. The factors that are responsible for the shift of the glucose threshold need to be defined. This has been accomplished in experimental animals in the case of fasting hyperinsulinemia associated with pregnancy [16] and in the pancreatectomy model of type 2 diabetes [17]. Placental lactogen and prolactin induce β-cell GK sufficiently to explain the threshold shift in pregnancy and a mild hyperglycemia is probably responsible for inducing β-cell GK in the diabetes model causing a threshold shift.

The present deliberations are not meant to minimize the critical role of the liver and of hepatic GK in glucose metabolism and homeostasis. It has indeed been demonstrated that MODY2 patients have a hepatic glucose phosphorylation deficit [18]. But it is not known whether this defect is sufficient or necessary to explain the mild hyperglycemia of MODY2. The liver does not seem to have a threshold response to glucose in contrast to the β-cells which would explain the predominant impact of the latter on the glucose set point of the organism.

It is however theoretically possible that hepatic glucose metabolism demonstrably regulated by GK translates into threshold phenomena in pertinent

metabolic pathways including glycogenolysis, gluconeogenesis, glucose release, ammonia- and ureogenesis and importantly lipogenesis and ketogenesis. Such thresholds, if demonstrable, might be dependent on neurohumoral input with acetylcholine, (nor)adrenaline, insulin and glucagon as principal candidate modifiers. Such mechanisms could elevate hepatic glucose metabolism from a graded substrate driven system to a primarily threshold regulated system directly influencing the glucose setpoint of the whole body. According to the current knowledge hepatic GK determines glucose usage with a sigmoidal glucose concentration dependency governed by the hepatic enzyme content, by the kinetic constants of the enzyme and GKRP (GK regulatory protein) [19]. The GK dependent process shows its steepest concentration dependency at the inflection point of the enzyme between 4–6 m*M* glucose, behavior which is at first glance unsuited for a direct GK dependent hepatic glucose threshold. Yet the possibility of a hepatic glucose threshold has not been systematically considered to our knowledge and it remains thus theoretically attractive to explore it by a comprehensive survey using state of the art liver cell preparations according to Berry and Friend [20] or physiologically more sophisticated approaches as pioneered by Cherrington and his colleagues [2]. In the meantime our thinking must be governed by the view that hepatic intermediary metabolism shows graded glucose dependency delegating the precise control of the basal blood sugar level to the β-cell.

This short essay had the objective to sketch the central role of the glucokinase system in blood sugar regulation briefly reflecting on established mechanisms by proposing a minimal mathematical model and floating a few trial balloons of speculation to help advance this exciting field.

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Franz M. Matschinsky, MD, Diabetes Research Center,
University of Pennsylvania School of Medicine,
501 Stemmler Hall, 36th & Hamilton Walk, Philadelphia, PA 19104-6015 (USA)
Tel. +1 215 898 4365, Fax +1 215 898 2178, E-Mail matsch@mail.med.upenn.edu

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Molecular Modeling of Human Glucokinase

Implications for the Activity of Mutants

Irene T. Weber [a], *Robert W. Harrison* [a], *Elizabeth Davis* [b], *Antonio Cuesta-Muñoz* [b], *Franz M. Matschinsky* [b]

[a] Department of Microbiology and Immunology, Thomas Jefferson University, Philadelphia Pa.,
[b] Diabetes Research Center, University of Pennsylvania, Philadelphia Pa., USA

Mutations in glucokinase are implicated in the development of type 2 diabetes. We have built a model of the structure of glucokinase with its substrates, glucose and ATP, in order to understand the effects of mutations on the enzyme activity. Glucokinase shares 30% identical amino acid residues with yeast hexokinase. This similarity was used to model the glucokinase structure based on the known crystal structure of yeast hexokinase with a sugar analog inhibitor. Yeast hexokinase undergoes a large conformational change on substrate binding from an open form in which the two domains are further apart to a closed conformation in which the two domains are closer together. Glucokinase is expected to show similar structural changes. The first models of the open conformation of glucokinase with sugar substrates were used to investigate the effect of mutations on the phosphorylation of sugars. Good agreement was obtained between predictions and the measured specificity for sugar substrates [17]. The activity of different mutations was analyzed with respect to their location on the model. Mutations of residues forming the substrate-binding site dramatically reduced the catalytic activity, as did mutations that were predicted to alter the structure. Other mutations located far from the active site showed only small reductions in activity. Recently, we have studied the role of ATP using a new model of glucokinase in the closed conformation complexed with both glucose and ATP. Mutations of residues close to the predicted ATP-binding site showed dramatic changes in the K_M for ATP and in the catalytic rate. This agreement confirmed our

model for ATP binding. This new model will be valuable for understanding the role of glucokinase mutations involved in diabetes.

Glucokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) is a 50 kD enzyme that catalyzes the ATP-dependent phosphorylation of glucose to glucose-6-phosphate as the first step, and the first rate-limiting step, in the glycolytic pathway. The family of related enzymes, glucokinase and hexokinases I, II and III, mediate glucose phosphorylation in mammalian tissues. Glucokinase is characterized by a high K_M for glucose (5–8 m*M* compared to 20 to 130 μM for human hexokinase) and a relative lack of product inhibition by glucose-6-phosphate compared to the hexokinases. Glucokinase has an important role as glucose sensor and metabolic signal generator in pancreatic β cells and hepatocytes [1–3]. A small decrease in the activity of glucokinase due to mutation will shift the threshold for insulin secretion in response to physiological glucose levels of 5 to 6 m*M*.

Mutations in the glucokinase gene can lead to development of an autosomal dominant form of noninsulin-dependent diabetes mellitus (NIDDM) [4–6]. More than 100 of these mutations have been identified. Glucokinase mutations associated with hypoglycemia and diabetes show a variety of kinetic defects, including reduced catalytic activity, increased K_M for glucose [7, 8] and/or increased K_M for ATP [9]. Other mutants show reduced thermal stability [9]. Recently, a glucokinase mutation was identified in a family with hyperinsulinemia [10]. This V455M mutant shows a decreased K_M for glucose [11].

Hexokinase isozymes also phosphorylate glucose in yeast cells. Yeast hexokinase is a paradigm for catalysis by induced fit: a large conformational change occurs on binding of glucose, as illustrated by two different crystal structures [12]. The crystal structures show that hexokinase folds into two domains, with the active site lying in a cleft between the two domains. The B isozyme of hexokinase was crystallized with the inhibitor, o-toluoylglucosamine (OTG), and the structure is in an 'open' conformation in which the two domains are further apart [13]. Yeast hexokinase A was crystallized with glucose. The structure is in a 'closed' conformation in which the two domains are closer together and glucose is buried in the active site cleft [14]. Similar conformational changes are expected to occur in human glucokinase when glucose binds.

The amino acid sequences of human glucokinase and yeast hexokinase are related by about 30% identity and the active site residues are conserved. This similarity has enabled us to build models of the glucokinase structure with its sugar substrates [15–17]. The model structures have been used to understand how glucokinase binds its substrates and to explain the molecular basis for the effects of mutations involved in diabetes [8, 18]. The new model presented here for the closed conformation of glucokinase in complex with

both glucose and ATP has provided good structural explanations for all the mutations with significant changes in kinetic parameters. The predicted ATP-binding site has particularly good agreement with the observed changes in K_M for ATP. Only a few mutations with small changes in kinetic parameters remain difficult to explain on the basis of the structural models.

Materials and Methods

Modeling the Open Conformation of Glucokinase with Glucose

The crystal structure of yeast hexokinase isozyme B was used to model the structure of human glucokinase. The atomic coordinates for the B isozyme were refined to an R-factor of 0.20 with 2.1 Å X-ray diffraction data and included the correct amino acid sequence [12]. A structural alignment was made for the amino acid sequences of human glucokinase and yeast hexokinase, which allowed deletions and insertions only between the elements of secondary structure. The amino acid residues of hexokinase were replaced by those of glucokinase, and a fragment search method was used to build the insertions and deletions [15]. The glucose was defined by the position of the OTG inhibitor in the hexokinase crystal structure. Subsequently, energy minimization with the program AMMP [19] was used to improve the model and incorporate glucose and solvent molecules from the hexokinase crystal structure [17]. This model was used to calculate the interaction energy for four different sugar substrates and had good agreement with the observed catalytic efficiencies for sugar phosphorylation.

Modeling the Closed Conformation of Glucokinase with Glucose and MgATP

The closed conformation of human glucokinase was modeled using the crystal structure of yeast hexokinase A as a guide. This crystal structure was determined at the low resolution of 3.5 Å and is not as accurate as the structure of the B isozyme. The model of glucokinase in the open conformation with glucose and solvent was minimized toward the structure of the closed conformation by restraining the positions of Cα atoms and of all side chains around the glucose-binding site. These features are all available in AMMP. The minimization was performed on a DEC Alpha Station 233. The ATP-binding site has not been identified in the hexokinase crystal structures. However, the crystal structure of the ATPase fragment of the heat-shock cognate protein, HSC70, with bound ADP [20] was used to model ATP in complex with brain hexokinase, as described in [21]. This ATP was combined with the model of the closed conformation of human glucokinase with glucose. When the tertiary complex was examined, an improved site for the adenine moiety of ATP was discovered in a pocket formed by residues that included the sites of mutations that alter the K_M for ATP. The adenine was manually rotated into this pocket, keeping the phosphates fixed, using the program CHAIN [22] on a Silicon Graphics Indigo2 workstation. This new model was minimized with AMMP and is described here.

Enzyme Kinetics Measurements

Human glucokinase and its mutants were expressed as fusion proteins with glutathione S-transferase (GST) as described in [9]. The wild-type glucokinase fusion protein was ki-

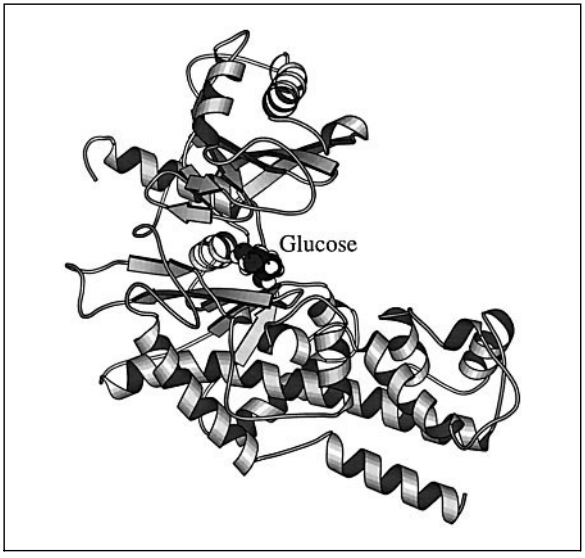

Fig. 1. Model structure of the open conformation of glucokinase with glucose. Glucokinase is shown in a ribbon representation with coils for alpha helices and arrows for beta strands. The glucose is shown with spheres for each atom.

netically indistinguishable from the enzyme purified without the GST fusion. The kinetic measurements are described in [11].

Results and Discussion

Model of Glucokinase in the Open Conformation with Glucose

Glucokinase is predicted to form a two-domain structure similar to that of the related enzyme, yeast hexokinase (fig. 1). The most conserved regions are the hydrophobic interior of the protein and the cleft between the two domains. The glucokinase model is expected to be relatively accurate for these conserved regions, as deduced by analysis of a similar model for HIV protease [23]. In contrast, the model is unlikely to be accurate for the regions of the 10 insertions and deletions compared to yeast hexokinase [15]. Fortunately, these regions of low similarity with hexokinase are all distant from the active site. The accuracy of the model has been verified by site-directed mutagenesis [16, 25] and correlation with the sugar specificity of glucokinase [17].

Glucose is predicted to bind in a deep cleft between the two domains, as shown in figure 1. The glucose-binding site is formed by glucokinase residues

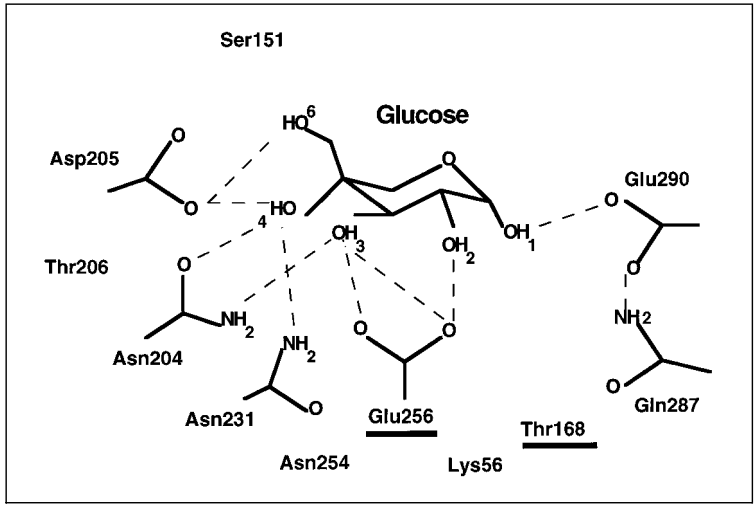

Fig. 2. Predicted interactions of glucokinase with glucose. Glucokinase residues that interact with glucose are shown. Hydrogen bond interactions are indicated by dashed lines. Sites of mutations T168P and V203A that alter the $S_{0.5}$ for glucose are underlined.

151–153, 166–169, 204–206, 225–231, 254–258, 287 and 290. Most of the residues that form the binding site for glucose are identical in both glucokinase and yeast hexokinase. The only exceptions are glucokinase residues Asn 166 and Cys 230. All the hydroxyl groups of glucose are bound by hydrogen bond interactions with the side chain atoms of glucokinase residues Asn 204, Asp 205, Asn 231, Glu 256 and Glu 290 (fig. 2). Indirect and water-mediated interactions are predicted with Lys 56, Ser 151, Thr 206, Asn 254, Gln 287 and 257–258 [16, 17]. Previously, the role of Asp 205 as catalyst in the reaction was confirmed [24].

The model for the glucose-binding site was used to identify residues for mutational analysis. Substitution of Asn 204 by Ala, Asp or Gln, of Glu 256 by Ala, and of Glu 290 by Ala resulted in more than 100-fold decrease in k_{cat}/K_M values compared to those of the wild-type enzyme. These changes are consistent with the predicted hydrogen bond interactions with glucose [16]. Lys 56 was predicted to have an indirect effect via an ionic interaction with Glu 256, and substitution of Ala for Lys 56 resulted in a two-fold increase in K_M for glucose with little effect on k_{cat}. Interestingly, mutational analysis of Ser 151 resulted in significant lowering of the K_M for glucose by as much as 40-fold for the Gly substitution [25]. In addition, the K_M for ATP was increased, and the ATPase activity was abolished. These results demonstrated the key importance of Ser 151 for catalysis.

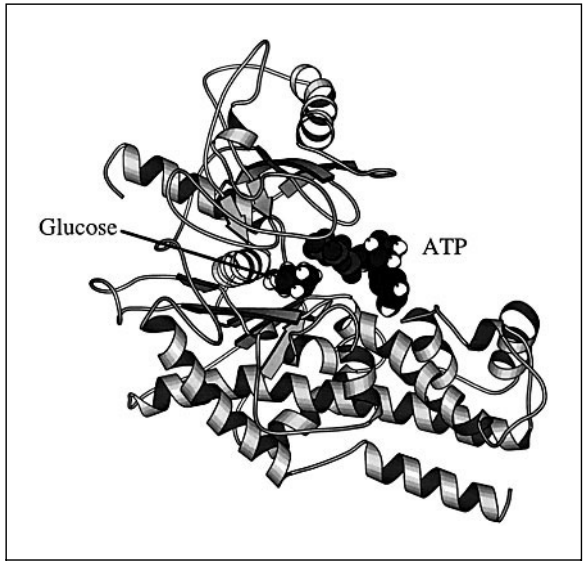

Fig. 3. Model structure of the closed conformation of glucokinase with glucose and ATP. Glucokinase is shown in a ribbon representation with coils for alpha helices and arrows for beta strands. The glucose and ATP are shown with spheres for each atom.

Correlation of Molecular Model with Sugar Specificity

Glucokinase phosphorylates different sugars with catalytic efficiencies in the order of glucose $\sim$ mannose $>$ deoxyglucose $>$ fructose [17]. The sugar specificity was analyzed by molecular mechanics calculations on the open conformation of glucokinase in complex with each of the four sugar substrates. It was important to include the crystallographic solvent from yeast hexokinase in this calculation due to the many water-mediated interactions with the sugar substrates. The interaction energy calculated for the glucokinase-sugar complexes gave an impressive correlation coefficient of 0.99 with the free energy differences derived from kinetic data [17]. Calculations on the model for the closed conformation also gave high correlation (unpublished data). Note that the calculated interaction energy is an estimate of the changes in internal energy of the enzyme-substrate complex, and does not include any estimate of entropic differences or changes in solvation. The good agreement with kinetic data verified the accuracy of the model for the sugar-binding site of glucokinase. These molecular mechanics calculations have also shown agreement with kinetic data for peptide substrates of retroviral proteases [26, 27].

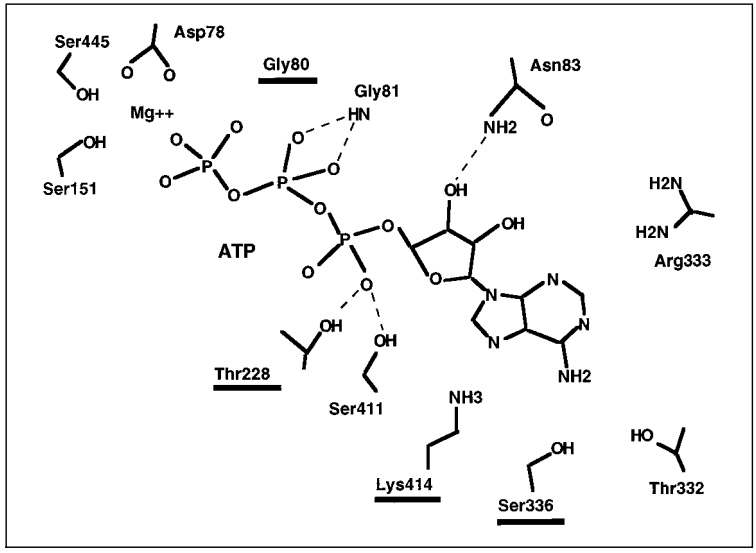

Fig. 4. Predicted interactions of glucokinase with ATP. Glucokinase residues that interact with ATP are shown. Hydrogen bond interactions are indicated by dashed lines. Sites of mutations G80A, T228M, S336L and K414E, that alter the K_M for ATP are underlined.

Model of Glucokinase in the Closed Conformation with Glucose and ATP

The new model represents the closed conformation of glucokinase with both substrates, glucose and MgATP, as shown in figure 3. The accuracy of this model is limited by the low resolution (3.5 Å) of the crystal structure of the closed conformation of yeast hexokinase. In future, we plan to verify the predicted structure by mutational and crystallographic analysis. However, the new model has already given an excellent explanation of the increased K_M for ATP observed for several mutants. The glucose is buried deeply in the cleft between the two domains, while the ATP is bound on the surface of the cleft and interacting with residues from both domains. The ATP-binding site is formed by glucokinase residues from both domains, 78–83, 151–152, 205, 227–228, 331–333, 336–337, 410–412, 415, 416 and 445. The magnesium ion and the phosphates of ATP are near the glucose and catalytic Asp 205. ATP is predicted to form hydrogen bond interactions with residues Gly 81, Thr 228, Ser 411 and Asn 83 (fig. 4). It is probable that additional interactions are mediated by water molecules. The magnesium ion is predicted to coordinate the gamma-phosphate of ATP and the amino acid side chains of Asp 78, Ser 445 and possibly Ser 151. As noted previously, Ser 151 is essential for ATP hydrolysis [25]. The predicted ATP-binding residues include sites of mutations that alter the K_M for ATP (fig. 4).

Correlation of Models with Activity of Glucokinase Mutants

The glucokinase mutations associated with diabetes and hypoglycemia have been studied enzymatically and the effects analyzed with respect to the new model of glucokinase with glucose and ATP. Previously, structural analysis using the model of glucokinase with glucose suggested that mutations located in the glucose-binding site (V203A, T228M, E256K, W357R), and mutations predicted to distort the structure (G261R and L309P) produced a dramatic reduction in catalytic activity [7, 8]. New kinetic data for many of the known mutants include more accurate measurements of the K_M for ATP [11]. The results have been interpreted in light of the model for glucokinase in complex with both glucose and ATP (figs. 3, 4). The structural analysis of kinetic data is summarized in table 1. Overall, mutants that show a large increase in $S_{0.5}$ for glucose of 4- to 120-fold also show an increased K_M for ATP (G80A, M210T, C213M, G261R, T168M and V203A). This effect on both glucose and ATP is probably because the enzyme must first bind glucose and undergo the conformational change to the closed form before the ATP can bind (figs. 1, 3). The only exception is V182M which had less than 3-fold decrease in K_M for ATP.

The mutations have been categorized by analysis of the models and kinetic data. All the mutations that increase the $S_{0.5}$ for glucose or the K_M for ATP by more than 4-fold are predicted to have structural connections either to the glucose- or the ATP-binding sites. One mutation shows a large decrease in k_{cat} only and is predicted to distort the secondary structure. The other mutations show changes of no more than 3-fold in any kinetic parameter, $S_{0.5}$ for glucose, K_M for ATP, or k_{cat}. Altered inhibition by the regulatory protein was ruled out for the mutants A53S, H137R, E300K and V367M. Some of these mutants show thermal instability, the rest are difficult to explain at this time.

Mutations with More than 4-Fold Increased $S_{0.5}$ for Glucose

1. *Residues in the Glucose-Binding Site*

Thr 168 and Val 203 are in or near the glucose-binding site (fig. 2). Thr 168 lies close to glucose and mutation to Pro has a dramatic effect on catalysis. Val 203 has a secondary effect via Asn 204 which forms a hydrogen bond interaction with glucose. Therefore, the mutation to Ala has a smaller effect on catalysis compared to T168P.

2. *Change Transmitted to the Glucose-Binding Site*

The predicted effect of these mutations is either to impede the conformational change induced by glucose or to transmit a small structural distortion

Table 1. Structural analysis of kinetic data for glucokinase mutants

Mutant	$S_{0.5}$ (m*M*) Glucose	K_M (m*M*) ATP	k_{cat} (sec-1)
WT	8.3	0.31	61.7
Change in glucose-binding site			
T168P	160.1	9.33	0.98
V203A	58.4	0.88	19.2
Change transmitted to glucose-binding site			
V182M	34.9	0.11	12.3
M210T	235	5.68	26.4
C213R	58.4	0.85	70.2
G261R	120	3.83	19.4
Change in ATP-binding site			
G80A	965	7.71	0.08
V226M	17.9	5.74	61.8
T228M	–	–	<0.02
S336L	5.4	21.7	5.4
K414E	12.3	1.73	46.7
Structural distortion			
L309P	9.5	0.58	2.9
Less stable			
H137R	7.5	0.24	45.3
E300K	10.3	0.46	59.0
V367M	7.6	0.31	65.2
Unexplained mechanism			
A53S	8.1	0.22	47.8
E70K	11.5	0.36	34.8
G175R	19.6	0.45	23.0
Hypoglycemic			
V455M	3.2	0.25	59.6

to the glucose-binding site that reduces the affinity for glucose. Each site of mutation is indirectly connected in three dimensions to the glucose-binding site. The mutated residues lie two residues away from the bound glucose so that the indirect action of the mutations on glucose is similar to pushing a row of dominoes. The side chain of Val 182 is adjacent to the side chains of Met 202 and Phe 148. This arrangement permits transmission of structural

changes to Asn 204 and Ser 151 (fig. 2), which have been shown to be important for glucose affinity and catalysis [16, 25]. Met 210 and Cys 213 lie on the same helix as the catalytic Asp 205, suggesting that mutants M210T and C213R have an indirect effect on glucose binding and catalysis. This explanation is consistent with the larger effect of M210T on catalysis compared to that of C213R, since Met 210 is closer to Asp 205. Mutation G261R is predicted to exert an indirect effect via the adjacent side chain of Tyr 289, which is next to Glu 290 that forms a hydrogen bond interaction with glucose (fig. 2).

Mutations with More than 5-Fold Increased K_M for ATP

Mutations G80A, V226M, T228M, S336L and K414E are predicted to directly alter the ATP-binding site (fig. 4). The individual kinetic effects of these mutations can readily be explained by the model structure. Gly 80 lies close to the magnesium ion and the gamma phosphate of ATP, so mutation to Ala is predicted to sterically hinder the binding of MgATP, resulting in the observed dramatic loss of activity. The hydroxyl side chain of Thr 228 is predicted to form a hydrogen bond interaction with the alpha phosphate of ATP, and mutation to Met would eliminate this interaction as well as introducing a larger side chain to block ATP binding, resulting in the lowest measured activity. Ser 336 lies at the bottom of the pocket where the adenine ring is predicted to bind, so mutation to the larger Leu side chain is expected to interfere with binding of ATP, consistent with the observed high K_M for ATP and low activity. However, this residue is relatively far from the glucose, in agreement with a relatively small effect on the $S_{0.5}$ for glucose. Lys 414 is predicted to lie adjacent to the adenine ring and far from glucose, so that mutation to Glu would alter the optimal shape and charge of the binding site, consistent with the observed smaller changes in K_M for ATP, $S_{0.5}$ for glucose, and k_{cat}. Finally, Val 226 is not predicted to interact directly with ATP, however, mutation to Met is expected to alter the position of Thr 228 and interfere with its critical interaction with ATP. This location is consistent with the observed smaller effect on catalysis.

Mutations with Large Change in k_{cat} Only

Mutations Predicted to Distort the Structure

L309P introduces the helix-breaking Pro into a helix far from the glucose- and ATP-binding site. This mutation is predicted to distort the secondary

structure without changing the substrate-binding sites, consistent with the observed 20-fold reduction in k_{cat}.

Mutations with Less than Two-Fold Change in Kinetic Parameters

1. *Mutations that Lower the Stability of the Enzyme*

Mutants H137R, E300K and V367M were shown to have reduced thermal stability compared to the wild-type glucokinase [9, 11].

2. *Mutations of Unexplained Mechanism*

Finally, the relatively small kinetic effects of four mutations remain difficult to explain on the basis of our structural models. One of these unexplained mutations is V455M, which is associated with hypoglycemia [10].

Conclusions

A new model has been built of glucokinase in complex with both substrates, glucose and MgATP. The glucokinase was modeled in a closed conformation with glucose bound in a deep cleft between the two domains. ATP is predicted to bind on the surface of the cleft where it interacts with residues from both domains of glucokinase. The magnesium ion is coordinated by the gamma phosphate of ATP, which lies near the glucose and the catalytic Asp 205. Analysis of this model has provided a structural explanation for all the glucokinase mutations with large changes in the kinetic parameters, $S_{0.5}$ for glucose, K_M for ATP, and k_{cat}. The new kinetic data for the mutants with altered K_M for ATP were in excellent agreement with the predicted location of the ATP in glucokinase. Mutants with large changes in the $S_{0.5}$ for glucose were predicted to act either directly on the glucose-binding site, or indirectly by transmission of small structural changes to the glucose-binding site. The analysis suggests that sugar phosphorylation by glucokinase is very sensitive to the exact conformation of the glucose- and ATP-binding sites. One mutation was predicted to distort the overall structure, without changing the substrate-binding sites. Some mutations with smaller effects on kinetic parameters can cause hyperglycemia by instability of the enzyme. Other mutations with small kinetic changes produce hypo- or hyperglycemia by some, as yet unknown, mechanism. In future, the new glucokinase model will be tested by mutational and crystallographic analysis.

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Franz M. Matschinsky, MD, Diabetes Research Center,
University of Pennsylvania, School of Medicine,
501 Stemmler Hall, 36th & Hamilton Walk, Philadelphia, PA 19104-6015 (USA)
Tel. +1 215 898 4365, Fax +1 215 898 2178, E-Mail matsch@mail.med.upenn.edu

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Cell-Specific Roles for Glucokinase in Glucose Homeostasis and the Pathogenesis of Hyperglycemia in MODY2 as Determined by Studies in Mice

Catherine Postic, Mark A. Magnuson

Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, Tenn., USA

Glucokinase (GK) gene mutations cause a monogenic form of diabetes mellitus known as maturity onset diabetes of the young, type 2 (MODY2). To determine the pathophysiological basis of the hyperglycemia that occurs in MODY2, we studied the consequences of either augmenting or reducing GK gene expression in the mouse, using both global and cell-specific strategies. These studies have revealed that there is a reciprocal relationship between GK gene copy number and the blood glucose concentration and that diminished GK gene expression in both the liver and pancreatic β cell individually contribute to a disturbance of glucose homeostasis in MODY2.

In 1992, Froguel et al. discovered that mutations in coding sequences of the GK gene cause maturity onset diabetes of the young, type 2 (MODY2), a disease that is characterized by early onset and persistent hyperglycemia [1]. Prior to this, a great deal of information had already been learned about the biochemistry, physiology, and molecular genetics of this hexokinase. Indeed, because GK was widely considered to play an important role in glucose metabolism in both β cells and liver, it was an ideal candidate gene for diabetes [2, 3]. Since the discovery that GK gene mutations cause MODY2, even more knowledge has been gained about the sites of expression, regulation, and structural features of GK in order to understand the physiopathological basis of this form of diabetes. This information, particularly that gained in studies of gene-altered mice, has helped to explain how the haploinsufficiency of GK

is able to disturb glucose homeostatic mechanisms, thereby causing this form of diabetes mellitus. However, even though useful information has been obtained by these studies, we do not yet fully appreciate all the mechanisms that are involved.

GK gene expression is cell-type specific and involves the use of alternate promoters [4]. The upstream promoter directs expression of this enzyme to pancreatic β cells and several other rare neuroendocrine cells in the brain and gut [5] whereas the downstream promoter is necessary for expression in the liver [6]. In pancreatic β cells, GK determines rates of glucose metabolism and thus is necessary for normal glucose-stimulated insulin secretion. In the brain, GK is found in several hypothalamic nuclei that are known either to affect feeding behavior or counter-regulatory responses. In the gut, the enzyme is expressed in neuroendocrine cells that secrete enteroincretins such as GLP-1 [5]. In liver, GK is thought to determine rates of both glucose uptake and glycogen synthesis, and to be essential for the regulation of various glucose-responsive hepatic genes [7].

The hyperglycemia of MODY2 is generally viewed as being due to diminished GK in pancreatic β cells, although a role for the liver has also been advocated by some investigators [8–13]. However, because GK is expressed in multiple cell types, and since the interactions that occur in vivo between various cell types are complex, the actual pathophysiological basis of MODY2 remains incompletely defined. Thus, to further explore the cell-specific roles of hepatic and β cell GK in glucose homeostasis, we have made use of both transgenic and Cre-*loxP* gene knock-out strategies in mice. Together, these studies allowed the pathophysiological effects of mutations within the GK gene to be more clearly defined.

Overexpression of GK

The effects of increased GK on blood glucose homeostasis were explored using transgenic mice that contained additional copies of the entire mouse GK gene locus [14]. An 83 kb transgene fragment was used that contains all the necessary DNA sequence information for the expression of both upstream (neuroendocrine-specific) and downstream (liver-specific) promoters [15]. Pronuclear DNA microinjection experiments yielded a line of transgenic mice that contained a single extra copy of the entire GK gene locus, thereby allowing the effects of increased GK gene copy number on glucose homeostasis to be directly examined. In this line of mice, GK mRNA concentration was increased by $\sim$1.5 fold in heterozygous transgenic animals and by $\sim$2 fold in homozygous animals [14].

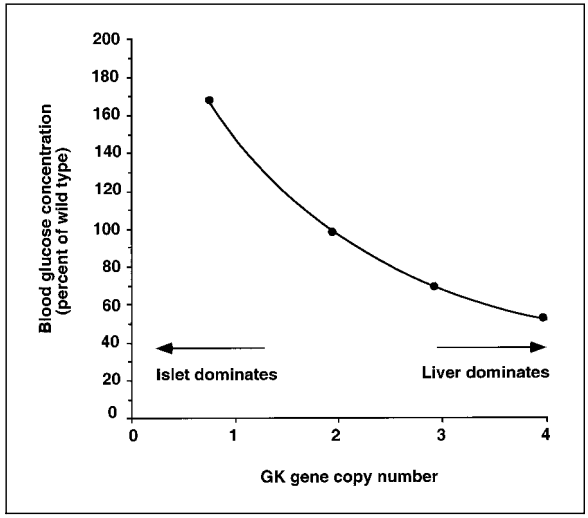

Fig. 1. Relationship between GK gene copy number and the blood glucose concentration. Resuls of GK overexpressing transgenic mice and total GK knock-out experiments are summarized. Total GK null mice have blood glucose concentration >500 mg/dl and do not survive the neonatal period.

Characterization of mice with a single extra copy of the GK gene locus revealed that they had a plasma glucose concentration 25% lower than littermates with 2 normal alleles (fig. 1). By backcrossing these mice, animals with a total of four functional copies of the GK gene were obtained. The plasma glucose concentration of these mice was reduced even further (fig. 1). By performing both basal and hyperglycemic clamp studies (using the protocol shown in fig. 2), we were able to precisely ascertain the effect of increased GK gene number on whole body homeostasis [16]. GK transgenic mice had a 21% increase in glucose clearance rate under basal conditions in the absence of any difference in basal insulin concentrations [16]. This finding indicates an essential role for GK in determining the basal rate of glucose clearance by the liver. Under hyperglycemic conditions, GK transgenic mice had glucose turnover and clearance rates similar to controls, but secreted $\sim 50\%$ less insulin (table 1). Interestingly, despite diminished insulin secretion, hepatic glycogen content was markedly increased after the 2-hour glucose infusion thereby also revealing a pivotal role for hepatic GK in controlling glycogen synthesis in liver (table 1). Islet GK expression was not increased in the GK transgenic mice, as would have been expected from the increased gene copy number. Instead, they had a diminished amount of the enzyme in islets, presumably

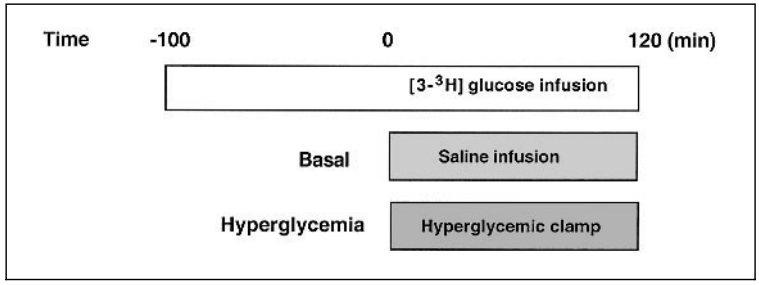

Fig. 2. Protocol for basal and hyperglycemic clamp studies. Basal and hyperglycemic clamp studies were performed using chronically cannulated, conscious, 6–8 h fasted mice as previously described [16]. The experimental protocol was 220-min in duration and consisted of a 100-min equilibration period (–100 to 0 min) followed by a 120 min experimental period. For basal studies saline solution was infused. For hyperglycemic clamp studies, a variable infusion of 50% glucose was used to raise blood glucose levels to ~300 mg/dl. A 2- to 4-μCi bolus of [3-^{3}H] glucose was given at –100 min, followed by a constant 0.04-μCi/min for the duration of the study (120 min) to determine glucose turnover rates and glycogen synthesis.

Table 1. Summary of metabolic parameters from basal and hyperglycemic clamp experiments

	Basal				Hyperglycemic			
Mouse model	gk^3	$gk^{del/w}$	$gk^{lox/w}$ + *Rip-cre*	$gk^{lox/lox}$ + *Alb-cre*	gk^3	$gk^{del/w}$	$gk^{lox/w}$ + *Rip-cre*	$gk^{lox/lox}$ + *Alb-cre*
Blood glucose	–12%	+38%*	+23%*	+40%*	Raised to ~300 mg/dl			
Plasma insulin	nd	nd	nd	nd	–40%*	–70%**	–70%**	–70%**
Glucose turnover	nd	–50%*	nd	nd	nd	–67%*	–56%*	–60%*
Glycogen synthesis	–	–	–	–	+360%**	–90%**	–55%*	–88%**

The following mouse models were used: gk^3: transgenic mice overexpressing the entire GK gene locus (3 functional genes in all tissues); $gk^{del/w}$: heterozygous GK knock-out mice (1 functional gene in all tissues); $gk^{lox/w}$ + *Rip-cre:* heterozygous β-cell-specific GK knock-out mice (a single functional gene in the β cell, two elsewhere); $gk^{lox/lox}$ + *Alb-cre:* liver-specific GK knock-out mice (no hepatic GK, two functional genes elsewhere). For the hyperglycemic clamp studies, blood glucose concentrations were raised to ~300 mg/dl. Each group of mice was compared to its appropriate control and differences between parameters are expressed as percent of controls. Only significant values are indicated in the table. nd: Not statistically different. *$p < 0.05$, **$p < 0.001$.

due to the mild hypoglycemia in these animals which diminishes the amount of β cell GK [16].

Transgenic mice that overexpress GK in the liver have also been generated by other groups [17, 18]. The results of these studies, which are similar to ours, confirm a direct role for hepatic GK in helping to determine the basal plasma glucose concentration. Together, they provide very strong evidence that increased hepatic GK, by itself, is able to increase the rate of glucose clearance by the liver without an increase in insulin secretion.

Targeted Disruptions of the Glucokinase Locus

Information complementary to the GK gain of function studies has been obtained using gene targeting to partially or totally eliminate GK gene expression [19–21]. In all instances, heterozygous GK knock-out mice are hyperglycemic, thereby indicating that studies in the mouse provide a relevant model for MODY2. Furthermore, all of the heterozygous GK null mice generated showed defective insulin secretion in response to glucose [19–21]. Thus, these studies indicate that the haploinsufficiency of GK impairs insulin secretion, a finding consistent with the proposed role of GK as glucose sensor in the β cell.

Cre-*loxP* Gene Targeting Strategy

To further investigate the cell-specific roles of GK in glucose homeostasis we made use of the Cre-*loxP* strategy to generate mice with cell-specific alterations of GK expression. Conditional and inducible gene knock-outs offer a means of overcoming the limitations of conventional gene targeting strategies [22–26]. However, this strategy is more complex than a conventional gene knock-out since two components are necessary. First, a conditional gene locus must be made by inserting two or more *loxP* sites in a nonessential portion of the gene, usually within an intron, by gene targeting in ES cells. For our studies, *loxP* sites were inserted into the GK gene between exons 8 and 9 and downstream of exon 10, thereby generating the loxed GK allele (gk^{lox}) shown in figure 3. The second essential part of the Cre-*loxP* strategy is to generate transgenic mice that express Cre under the control of cell-specific promoters. For this purpose, we generated transgenic mice that express Cre under the control of either the insulin or the albumin promoters, which expressed this site-specific recombinase in pancreatic β cells and hepatocytes, respectively, as shown in figure 4. By crossing the gk^{lox} allele into mice bearing one or another

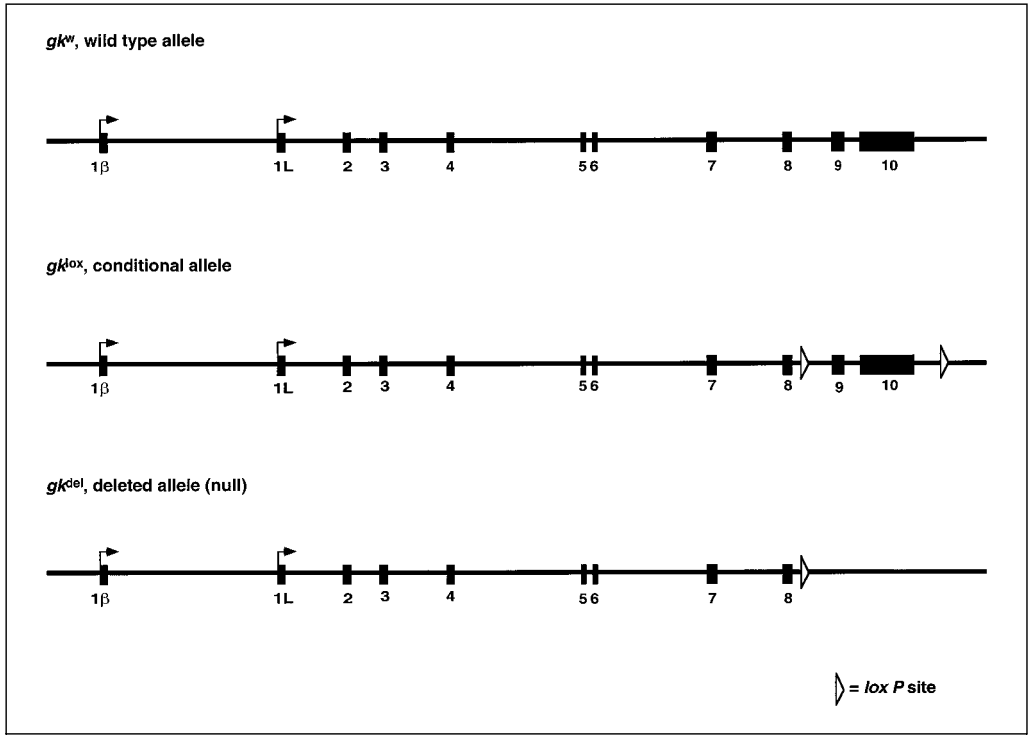

Fig. 3. Generation of mice with conditional *gk* gene alleles. Exons are indicated as solid rectangles. A partial map of the wild-type allele (gk^{w}) is shown. The conditional allele was obtained by homologous recombination in ES cells and partial Cre recombination (gk^{lox}) [27]. The deleted allele (gk^{del}) lacks exons 9 and 10 and was obtained by microinjection of a Cre expression plasmid into single-cell mouse embryos or by cell-specific expression of Cre in transgenic mice.

of the Cre transgenes, we were able to specifically eliminate GK in either β cells or liver. In addition, by microinjecting a Cre expression plasmid into single-cell mouse embryos, we were able to generate a null allele (gk^{del}), thereby generating mice that served to further confirm the effects of a global GK gene knock-out [27].

Global GK Null Mice

Heterozygous GK null mice ($gk^{del/w}$) generated by the Cre-*loxP* strategy were viable and had both fed (+75%) and fasting hyperglycemia (+40%)

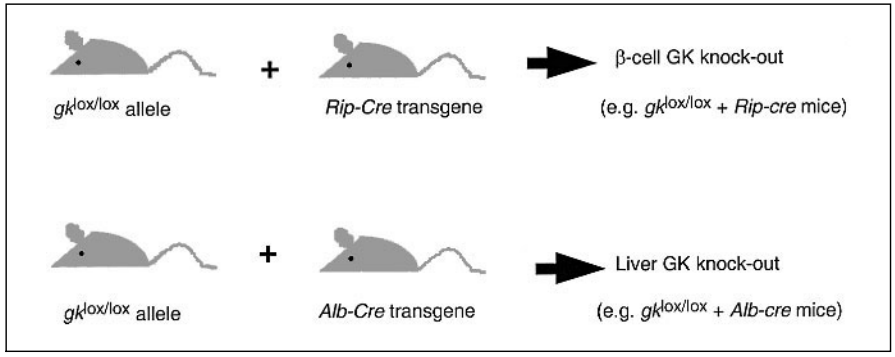

Fig. 4. Strategy for β-cell and liver-specific deletion of the gk^{lox} allele in mice. Two lines of Cre transgenic mice were created. *Rip-cre* transgenic mice express Cre in the pancreatic β cells under the control of the rat insulin promoter. *Alb-cre* transgenic mice express Cre in liver under the control of the promoter/enhancer albumin gene. Intercrossing Cre transgenic mice with gk^{lox} mice produces both β-cell and liver-specific GK gene knock-out animals.

compared to the control mice (fig. 1). Adult $gk^{del/w}$ mice had a ~50% reduction in basal glucose turnover rates (table 1). Marked glucose intolerance was also evident in these mice during a hyperglycemic clamp, as reflected by ~70% reduction in glucose turnover rates and a 60% decrease in the peak of insulin secretion compared to controls (table 1). At the end of the hyperglycemic clamp, newly synthesized hepatic glycogen was decreased by 90% in $gk^{del/w}$ mice compared to controls (table 1).

By intercrossing $gk^{del/w}$ mice with each other we obtained mice that were homozygous null for GK ($gk^{del/del}$) (table 2). These mice appeared to be normal at birth, but died within four days of birth of profound hyperglycemia. The metabolic alterations observed were all consistent with insufficient insulin secretion, presumably because of the failure of β cells to respond to an elevated plasma glucose concentration. Thus, the phenotype of our GK null mice closely resembled that of Grupe et al. [20], but was substantially different from that reported by Bali et al., whose animals died in utero at embryonic day 9.5 [19]. The possible reasons for the marked differences between these different lines of mice have been previously discussed [27].

β-Cell-Specific Knock-Out of GK

To more clearly determine the role of GK in pancreatic β cells, gk^{lox} mice were interbred with mice that expressed Cre under the control of the rat insulin

Table 2. Summary of GK overexpression and gene deletion experiments in the mouse

Type of manipulation	Summary of phenotype
Transgenic mice that overexpress both islet and hepatic GK	Mild hypoglycemia due to increased hepatic GK; β-cell GK is downregulated; no change in basal insulin concentrations
Homozygous null global GK knock-out mice ($gk^{del/del}$)	Severe hyperglycemia and death by postnatal day 4
Heterozygous null global GK knock-out mice ($gk^{del/w}$)	Moderate hyperglycemia; diminished insulin secretory response
Total β-cell-specific knock-out mice ($gk^{lox/lox} + Rip\text{-}cre$)	Severe hyperglycemia and death by postnatal day 4
Heterozygous β-cell-specific knock-out mice ($gk^{low/w} + Rip\text{-}cre$)	Moderate hyperglycemia; diminished insulin secretory response
Total liver-specific knock-out mice ($gk^{lox/lox} + Alb\text{-}cre$)	Mild hyperglycemia; impaired insulin secretory response to glucose; impaired glycogen synthesis

promoter (*Rip-cre*) [27] (fig. 4). The resulting mice had either a partial ($gk^{lox/w} + Rip\text{-}cre$) or nearly complete ($gk^{lox/lox} + Rip\text{-}cre$) knock-out of GK only in β cells. Mice with the total β-cell-specific knock-out of GK (e.g. $gk^{lox/lox} + Rip\text{-}cre$) died within a few days of birth as a result of severe diabetes (table 2). Plasma insulin concentrations in these animals were decreased by $\sim 70\%$ in two-day-old pups and they exhibited identical metabolic and biochemical alterations that were observed in GK-null neonates, namely steatosis and depletion of hepatic glycogen content. Thus, mice that lack GK only in β cells are phenotypically similar to animals with a global GK knock-out, as well as animals that lack only the islet isoform of GK [21].

Basal and hyperglycemic clamp studies of the heterozygous β-cell-specific GK knock-out mice (e.g. $gk^{lox/w} + Rip\text{-}cre$) showed they had fasting hyperglycemia ($+25\%$) without measurable difference in basal insulin levels (table 1). During the hyperglycemic clamp, glucose turnover rates and insulin secretion were reduced by $\sim 70\%$. As a consequence of diminished insulin secretion, net glycogen synthesis in liver was reduced by $\sim 50\%$ (table 1).

Liver-Specific Knock-Out of GK

To determine directly the role of hepatic GK in glucose homeostasis mice bearing a rat albumin promoter/enhancer-Cre transgene (*Alb-cre*) were made and crossed with mice bearing the loxed GK allele. In this manner, both

heterozygous ($gk^{lox/w} + Alb\text{-}cre$) and homozygous ($gk^{lox/lox} + Alb\text{-}cre$) liver-specific GK gene knock-out animals were generated (fig. 4). In contrast to the perinatal lethality associated with the lack of β cell GK, the total loss of hepatic GK did not cause the mice to die. Instead, mice totally lacking hepatic GK (e.g. $gk^{lox/lox} + Alb\text{-}cre$) had fed blood glucose values that were only ∼10% higher than their $gk^{lox/lox}$ littermates [27].

Again, to explore the consequences of the total loss of hepatic GK on both insulin secretion and hepatic fluxes, basal and hyperglycemic clamp studies were performed (table 1). After an 8-hour fast, no differences were detected in either plasma insulin concentration or glucose turnover rate, even though the blood glucose concentrations were higher (+40%) than the controls (table 1). During the hyperglycemic clamp phase of the study, glucose turnover rate was markedly reduced in the liver-specific GK knock-out mice (table 1). Surprisingly, the $gk^{lox/lox} + Alb\text{-}cre$ mice secreted 70% less insulin in response to the glucose stimulus compared to controls (table 1), a finding that is discussed in more detail below. Net glycogen synthesis was also reduced by ∼90% in liver (table 1). Together, these results clearly indicate that hepatic GK plays an important role in glucose homeostasis, by both a direct effect on hepatic glucose utilization and an indirect effect on insulin secretion.

Role of β-Cell GK in Glucose Homeostasis

It is now well established that glucose-stimulated insulin secretion is a multistep process that depends on increased metabolic fluxes within the β cells [28]. GK has been widely viewed as playing a key role in this process by serving as the pancreatic β-cell glucose sensor, as first proposed by Matschinksy et al. [29]. Studies of both global and pancreatic β-cell-specific GK gene knock-out mice have now clearly established that the haploinsufficiency of GK causes a glucose-sensing defect, which is manifested by diminished insulin secretion [19–21, 27]. Even more direct evidence for the function of GK as the β-cell glucose sensor was recently obtained by Piston et al. [30]. By isolating islets from $gk^{lox/lox}$ mice and treating them with a recombinant adenovirus that expresses Cre, we were able to remove GK from some β cells, while retaining it in others. Two photon excitation microscopy of these islets showed that individual β cells that lack GK have an attenuated rise in NADP(H) autofluorescence, in contrast to cells that contain GK [30].

Previously, Terauchi et al. [21] have shown that mice that are deficient in just the islet isoform of GK are hyperglycemic. This finding has also been interpreted as indicating that the haploinsufficiency of GK in the pancreatic β cell is the main reason for hyperglycemia in MODY2. However, since the

islet isoform is also expressed in the brain and gut, it was possible, at least in principle, that GK in certain brain nuclei and in certain gut enteroendocrine cells was playing a major role in the pathogenesis of hyperglycemia. While additional studies are needed, it is clear from the β-cell-specific knock-outs of GK that extrapancreatic sites of GK, which are presumed also to play a role in glucose sensing, are incapable of overriding the defect in β cell caused by the loss of one functional GK allele.

Interestingly, despite the close similarities between mice and humans in the mechanisms used to maintain glucose homeostasis, the loss of one functional GK allele in mice results in a greater degree of hyperglycemia than is observed in humans. Recently, Sreenan et al. [31] have shown that islets from heterozygous GK knock-out mice may be protected against the toxic effect of high glucose through enhanced expression of the remaining normal allele. These studies provide evidence for an adaptation of heterozygous GK null mice to hyperglycemia, and thus may help to explain why MODY2 in humans does not usually become more severe with time.

Role of Liver GK in Glucose Homeostasis

It has previously been difficult to directly assess the role that a deficiency of hepatic GK plays in the pathogenesis of MODY2. While it has been widely assumed that GK is necessary for hepatic glucose disposal, the complete loss of hepatic GK is not lethal, and in fact has only a relatively small effect on the plasma glucose concentration (+ 10–40% depending on dietary state). The fact that basal glucose turnover rates in mice totally lacking GK is similar to control animals suggests that flux through one or another of the low K_m hexokinases is sufficient in the basal state. The effect of a deficiency of hepatic GK becomes more apparent under hyperglycemic conditions. In this situation, glucose turnover, glucose clearance, and hepatic glycogen synthesis rates are all markedly decreased.

Previous studies provide some insights into the key role of GK in the regulation of hepatic glucose fluxes. In the rat, the selective inhibition of hepatic GK activity by the infusion of glucosamine has been shown to impair the ability of hyperglycemia to suppress endogenous glucose production [32]. Rossetti et al. have also reported that hyperglycemia fails to fully suppress hepatic glucose production in a heterozygous null global GK knock-out mouse [33]. These findings suggest that the haplodeficiency of GK in MODY2 may lead to a general disturbance in hepatic glucose metabolism, thereby limiting the ability of the liver to normally suppress glucose production rates in the face of hyperglycemia.

The haplodeficiency of hepatic GK in MODY2 might also contribute to impaired glucose homeostasis by exacerbating the defect in insulin secretion brought on the lack of β-cell GK. Indeed, it was surprising to find that mice that lacked hepatic GK, and which only had a small elevation in basal plasma glucose concentrations, showed markedly impaired insulin secretion during the hyperglycemic clamp. This finding can be interpreted in at least two different ways. First, it is possible that the mild hyperglycemia itself is sufficient to impair insulin secretion by the β cell. Sustained hyperglycemia, even mild [34–36], has been shown to cause β cells to become unresponsive to glucose, thereby leading to impaired glucose-induced insulin secretion [37, 38]. Second, it is possible that the lack of hepatic GK impairs the ability of the liver to sense and respond to hyperglycemia. If hepatic GK functions as a liver glucose sensor, similar to the role that islet GK has as a β-cell glucose sensor, then hyperglycemia may stimulate the secretion of yet to be defined factor or factors from the liver that act on the β cell to promote insulin secretion. Impaired secretion of such a hepato-incretin factor, presumably due to the lack of hepatic GK, could explain the attenuated insulin secretion in response to a hyperglycemic challenge that was observed in the liver-specific GK knock-out mice.

The finding that hepatic GK, in addition to that of β-cell GK, plays a role in causing the hyperglycemia of MODY2 is consistent with studies showing an impairment in liver glucose metabolism in patients with this disease. Abnormal regulation of hepatic glucose output and impaired glycogen synthesis have previously been reported in patients with MODY2 [39, 40]. Taken together, both the gain and loss of function studies that have been performed indicate that hepatic GK itself is a major determinant of glucose homeostasis.

Implications for the Pathogenesis of Hyperglycemia in Other Types of MODY

An understanding of the mechanisms by which diminished GK causes hyperglycemia may be important in elucidating how defects in the expression of other genes cause hyperglycemia and diabetes. In MODY2, a haplodeficiency of GK in both β-cell and hepatic GK conspire to cause sustained and pronounced hyperglycemia. Since several other MODY genes are also expressed in both the liver and islet, the pathogenesis of diabetes in response to a haplodeficiency of these genes may be similarly complex, and also involve impaired function of both the liver and β cell. For instance, HNF-1α [41], HNF-4α [42] and HNF-3β [43] are each expressed in the liver as well as the pancreatic islet. The only clear exception may be Pdx-1 since this gene has

only been found to be expressed in the pancreas and small intestine, and is not present in the liver [44, 45]. In any case, further studies will likely be required to determine how each different gene defect is able to interfere with normal glucose homeostatic mechanisms and thereby cause a diabetic phenotype.

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Mark Magnuson, MD, Vanderbilt University School of Medicine, 723 Light Hall,
Nashville, TN 37232-0615 (USA)
Tel. +1 615 322 7006, Fax +1 615 322 7236, E-Mail mark.magnuson@mcmail.vanderbilt.edu

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Control of Glucose Phosphorylation/ Dephosphorylation in the Liver

Emile Van Schaftingen, Maria Veiga-da-Cunha, Isabelle Gerin, Moulay Moukil

Laboratory of Physiological Chemistry, ICP and Université Catholique de Louvain, Brussels, Belgium

Glucokinase and glucose-6-phosphatase catalyse opposite reactions in the liver. Whereas the glucose-phosphorylating enzyme shuttles between the cytoplasm and the nucleus, glucose-6-phosphatase is an enzyme associated with the endoplasmic reticulum. Glucokinase activity is controlled not only by the concentration of glucose but also by a regulatory protein that binds to the enzyme in a fructose 6-phosphate and fructose 1-phosphate-sensitive manner. Studies with various 'effectors', or precursors of effectors, of glucokinase and of its regulatory protein suggest that the partition of this enzyme between the nucleus and the cytoplasm is largely dictated by its affinity for the regulatory protein, a nuclear protein. Site-directed mutagenesis studies indicate that the binding site for the regulatory protein is present on the smaller domain and in the hinge region of glucokinase, suggesting that the inhibitor may act by 'freezing' this enzyme in an open conformation.

Glucose-6-phosphatase is a transmembrane protein whose catalytic site faces the lumen of the endoplasmic reticulum. This topology makes that the enzyme needs transporters for glucose 6-phosphate, inorganic phosphate and glucose. Our group recently identified a gene encoding a putative glucose 6-phosphate translocase, which has now been found to be mutated in more than 30 patients with glycogen storage disease type Ib. This disease is known to be due to a lack of glucose 6-phosphate transport across the membrane of the endoplasmic reticulum.

Glucokinase and glucose-6-phosphatase catalyse opposite reactions in the liver. While the understanding of the short-term regulation of the first enzyme has witnessed important progress in recent years, the molecular constituents

of the glucose-6-phosphatase system are still in the process of being identified at the molecular level. The purpose of the present article is to review recent advances made along these two lines of research.

Glucokinase is a member of the hexokinase family, which, in contrast to other mammalian hexokinases, is characterized by a low affinity for glucose, by a sigmoidal saturation curve for this substrate and by being sensitive to inhibition by a regulatory protein [reviewed in 1–4]. Due to its low affinity for glucose, the activity of glucokinase can be modulated by millimolar concentrations of this hexose. Accordingly glucokinase is present in cell types that have to 'sense' the blood glucose concentration, i.e. the hepatocytes, the beta cells [1–4] and the alpha cells of pancreatic islets [5].

Glucokinase is a monomeric enzyme and remains a monomer even in the presence of its substrates [6]. Therefore, the sigmoidicity of the substrate saturation curve cannot be accounted for by classical allosteric models, in which positive cooperativity is based on the interaction between homologous subunits of an oligomeric protein. Because there does not appear to be a second binding site for glucose [7], kinetic models of cooperativity have been proposed to explain the sigmoidal behavior [8–9]. In such models, the positive cooperativity results from the existence of two conformations with different affinities for glucose. These two conformations are maintained far from equilibrium in the course of the catalytic cycle and their relative abundance depends on the glucose concentration.

The Regulatory Protein of Glucokinase

The regulatory protein of glucokinase was discovered as a result of the observation that fructose stimulates the phosphorylation of glucose in rat hepatocytes [10, 11]. This effect was found to be mediated by fructose 1-phosphate, which releases the inhibition exerted by a regulatory protein on glucokinase [12]. This protein was purified and shown to inhibit glucokinase, competitively with respect to glucose [12, 13], in the presence of fructose 6-phosphate although not in the presence of fructose 1-phosphate. Fructose 6-phosphate promotes, and fructose 1-phosphate inhibits, the association of the enzyme-regulatory protein complex [14], both by binding to the regulatory protein [15]. The fact that the inhibition is competitive with glucose [13] indicates that the regulatory protein and glucose do not bind simultaneously to the enzyme.

The first indication that the regulatory protein binds to a site distinct from the catalytic site came from kinetic studies, which showed that the regulatory protein and the glucose analogue N-acetyl-glucosamine act syner-

gistically and, therefore, bind simultaneously to the enzyme [13]. Mutations of glucokinase-specific residues [16, Moukil MA, Veiga-da-Cunha M, Van Schaftingen E, unpublished results] indicates that the binding site for the regulatory protein resides on the smaller domain and on the hinge region in the three-dimensional model of glucokinase proposed by St-Charles et al. [17]. This suggests that the regulatory protein inhibits glucokinase by preventing the glucose-induced conformational change.

The cDNA of the rat liver regulatory protein was sequenced and shown to encode a 626 residue polypeptide, with a predicted molecular mass of 68.5 kDa [18]; human regulatory protein shows 88% sequence identity with its rat liver counterpart [19]. Its gene contains 19 exons [20] and is located on chromosome 2p23 [19, 21]. The recent sequencing of bacterial genomes has disclosed the existence of open reading frames predicting proteins homologous to the liver regulatory protein in several bacteria including *Haemophilus influenzae* [22] and *Bacillus subtilis*. The function of these proteins is unknown; interestingly they show weak but significant identity with glucosamine 6-phosphate isomerase, an enzyme that converts fructose 6-phosphate and glutamine to glucosamine 6-phosphate and glutamate. The bacterial homologues of the regulatory protein may thus have affinity for fructose 6-phosphate or a closely related molecule.

Physiological Role (and Potential Pathophysiological Role) of the Regulatory Protein

A consequence of the existence of the regulatory protein is that liver glucokinase activity can be regulated by fructose 1-phosphate and fructose 6-phosphate. As mentioned above, the deinhibitory effect of fructose 1-phosphate accounts for the stimulatory effect of fructose on glucose utilization by the liver. Fructose can therefore act as a nutritional signal that favors glucose utilization when carbohydrates are present in the diet [3].

The role of the inhibitory effect of fructose 6-phosphate is most likely to decrease glucose phosphorylation when glycogenolysis and/or gluconeogenesis are stimulated. Stimulation of these two pathways raises the concentrations of glucose 6-phosphate and fructose 6-phosphate (which are maintained close to thermodynamic equilibrium thanks to the high activity of phosphoglucose isomerase). The increase in the glucose 6-phosphate concentration stimulates glucose 6-phosphatase, whose Km is relatively high compared to the intracellular substrate concentration [23], whereas fructose 6-phosphate inhibits glucose phosphorylation. Both effects add up to increase net glucose production by the liver.

However, the fact that fructose 6-phosphate indeed inhibits glucose phosphorylation in hepatocytes was difficult to demonstrate, because glucose 6-

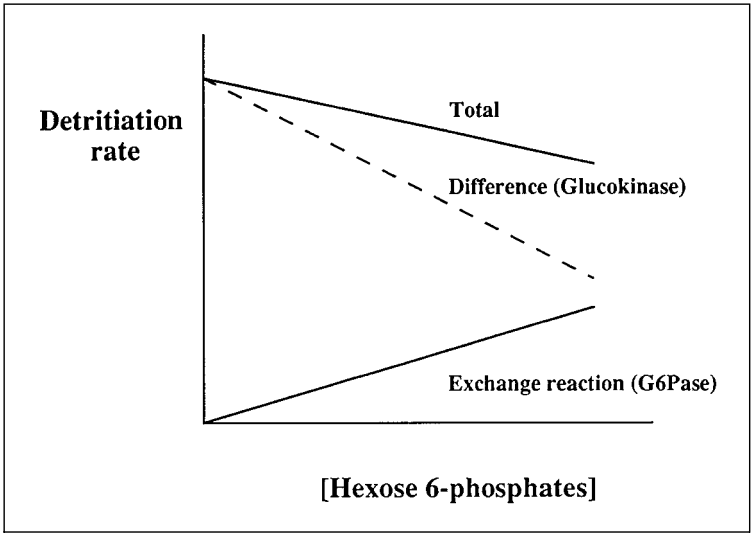

Fig. 1. Contribution of glucokinase and glucose-6-phosphatase to the detritiation of [2-^{3}H]glucose at different intracellular concentrations of hexose 6-phosphates. Glucokinase is inhibited by fructose 6-phosphate in the presence of the regulatory protein. Glucose-6-phosphatase catalyses an exchange reaction (glucose/glucose 6-phosphate) which also contributes to the conversion [2-^{3}H]glucose to [2-^{3}H]glucose 6-phosphate and tritiated water, and which is stimulated by glucose 6-phosphate.

phosphatase catalyses an exchange reaction between glucose 6-phosphate and glucose [24], as a result of the fact that its reaction mechanism proceeds via the formation of a phosphoryl-enzyme intermediate [25]. The exchange reaction depends on the glucose 6-phosphate concentration and hence is stimulated when the concentration of hexose 6-phosphates increases in hepatocytes (fig. 1), thereby masking the decrease in the net rate of glucose phosphorylation when the latter is estimated with radiolabelled glucose (most commonly by the conversion of (2-^{3}H)glucose to tritiated water). The contribution of the exchange reaction catalysed by glucose-6-phosphatase to the detritiation of (2-^{3}H)glucose could be estimated in isolated hepatocytes by shutting off glucokinase activity with the powerful inhibitor, glucosamine. Calculations derived from these data allowed us to conclude that glucokinase was indeed inhibited when the concentration of hexose 6-phosphates increased [26].

Further proof came from the finding that the rate of glucose phosphorylation is also decreased when hepatocytes are incubated in the combined presence of mannitol or sorbitol and SDI 158, an inhibitor of sorbitol dehydrogenase [27] that prevents the conversion of sorbitol (a contaminant

of mannitol, see below) to fructose and fructose 1-phosphate [28]. The inhibitory effect observed under these conditions could be attributed to mannitol 1-phosphate and sorbitol 6-phosphate, two analogues that act like fructose 6-phosphate on the regulatory protein [29] and can be formed in hepatocytes in a transphosphorylation reaction catalysed by glucose-6-phosphatase at the expense of glucose 6-phosphate [28]. These data further pleaded for the role exerted by fructose 6-phosphate.

The regulatory protein could play a role in diabetes if mutations happen to make it a 'super-inhibitor' of glucokinase. An example of such a 'super-inhibitory' protein is the regulatory protein of *Xenopus* liver, which inhibits glucokinase in the absence of fructose 6-phosphate and is insensitive to deinhibition by fructose 1-phosphate [30]. Since this protein is homologous to rat liver regulatory protein, it is not excluded that point mutations could make human regulatory protein insensitive to fructose-phosphates. Such a protein would inhibit glucose phosphorylation in the liver irrespective of the intracellular metabolite concentration and could therefore cause a dominant form of diabetes. No linkage could be found between maturity-onset diabetes of the young (MODY) diabetes and the regulatory protein gene in a limited number of families [21], but this should be reinvestigated as it is more and more apparent that MODY is heterogeneous.

Glucokinase Translocation

Loranne Agius first provided evidence for the translocation of glucokinase from one site to another in liver cells in culture. When hepatocytes that have been incubated in the presence of a low concentration of glucose (5 m*M*) are permeabilized with digitonin in a medium containing 5 m*M* $MgCl_2$, only minimal glucokinase release occurs. When, in contrast, the cells have been incubated in the presence of fructose, sorbitol, mannitol, an elevated concentration of glucose or mannoheptulose, a much larger release of glucokinase is observed. These effects are counteracted by agents such as ethanol, glycerol and glucosamine [31–33]. In the presence of $MgCl_2$, the regulatory protein is also only minimally released from hepatocytes by digitonin treatment, but in this case, pretreatment of the cells with fructose or an elevated concentration of glucose did not modify this release [34].

Immunohistochemistry experiments have now shown that these changes in the localization correspond to a transfer of glucokinase from the nucleus to the cytosol and that the regulatory protein is a nuclear protein [35–37], leading to the hypothesis that the regulatory protein serves as an anchor that maintains glucokinase in the nucleus under conditions where the two proteins

bind to each other (low glucose concentration, no fructose), but allows it to be transferred to the cytosol when it has low affinity for glucokinase.

The use of a very sensitive fructose 1-phosphate assay [38] has enabled us to show that there is an excellent correlation between the effects of fructose and sorbitol on glucokinase translocation and their ability to increase the fructose 1-phosphate concentration [39]. The effect of glucose is explained partly by its action to increase the fructose 1-phosphate concentration and partly by its own effect to cause dissociation of the glucokinase-regulatory protein complex. The latter explanation also applies to mannoheptulose, an inhibitor of glucokinase which was shown to act competitively with respect to the regulatory protein [39]. The stimulatory effect of mannitol on the translocation of glucokinase was readily explained by the presence of contaminating sorbitol in all mannitol preparations [39]. In marked contrast, mannitol exerts an inhibitory effect on glucokinase translocation in the presence of the inhibitor of sorbitol-dehydrogenase SDI 158 [28], most likely because the effect of mannitol 1-phosphate (which is formed from mannitol, see above) is no longer counteracted by fructose 1-phosphate. All these observations indicate that the translocation is promoted by agents that favor the dissociation of the glucokinase-regulatory protein complex by binding either to the regulatory protein (fructose 1-phosphate) or to glucokinase (glucose); furthermore, the translocation is inhibited by an agent (mannitol 1-phosphate) that favors the association of the glucokinase-regulatory protein complex (fig. 2).

What could be the role of glucokinase and of its regulatory protein in the nucleus? One should first say that mere transfer of glucokinase to the nucleus without binding to an inhibitory protein is not expected to shut off this enzyme's activity since the nuclear pores are large enough to allow rapid diffusion of nucleotides and most likely also of glucose. Direct interaction of glucokinase with an inhibitory protein is, therefore, important and translocation is most likely needed because the regulatory protein is a nuclear protein. What is then the role of the regulatory protein in the nucleus? One possiblility is that it participates, maybe together with glucokinase, in the regulation of gene expression. Alternatively the nuclear localization of these proteins only serves the purpose of regulating the enzymic activity by providing additional levels of control. In this respect, the nucleus is the only compartment where proteins can be both imported and exported.

Glucose-6-Phosphatase

It has been known since about 1950 that glucose-6-phosphatase is associated with the endoplasmic reticulum [40], and this localization is most likely

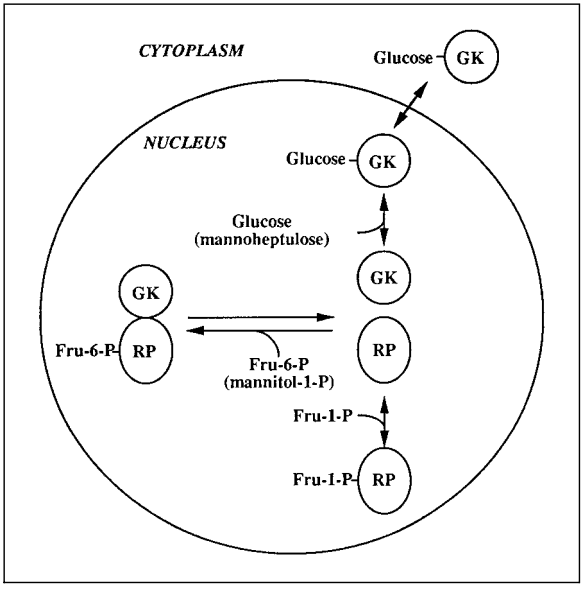

Fig. 2. Model for the regulation of glucokinase translocation by association of this enzyme with its regulatory protein. Fructose 6-phosphate and its analogue mannitol 1-phosphate favor the formation of the complex and inhibit translocation of glucokinase. Other agents favor translocation by causing dissociation of the complex, either by binding to glucokinase (glucose, the competitive inhibitor mannoheptulose) or to the regulatory protein (fructose 1-phosphate).

the main reason why the molecular characterization of glucose-6-phosphatase lagged behind that of glucokinase. The observation that the specificity of this enzyme is broader in detergent-treated microsomes than in intact microsomes, as well as other observations on the kinetic properties of this enzyme, led to the proposal that the catalytic site of this enzyme faces the lumen of the endoplasmic reticulum and that hydrolysis of glucose 6-phosphate requires the presence of transporters for glucose 6-phosphate, inorganic phosphate (Pi) and, possibly also glucose [41, 42] (fig. 3).

Glucose-6-phosphatase deficiency results in glycogen accumulation in the liver and in kidneys, and in hypoglycemia, lactic acidosis, hypertriglyceridemia and hyperuricemia (reviewed in 43). The most common form of this disease, known as glycogen storage disease type Ia (GSD Ia), is due to a deficiency in the hydrolase, resulting from mutations in the gene encoding this enzyme [44, 45]. About 20% of the cases with the clinical picture mentioned above have a normal glucose-6-phosphatase activity in detergent-treated microsomes

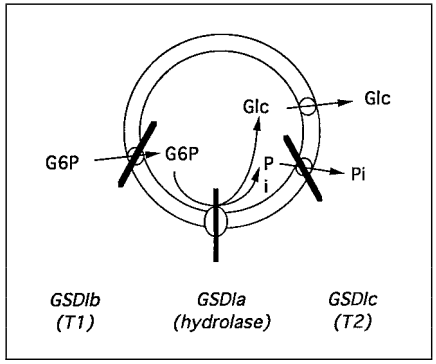

Fig. 3. The glucose-6-phosphatase system and the various forms of glycogen storage disease type I. See text. The translocases for glucose 6-phosphate, Pi and glucose are often named T1, T2 and T3.

but a reduced activity in intact microsomes, as a result of a deficiency in glucose 6-phosphate transport [46–48]. Interestingly, most of the patients with this condition, known as GSD Ib, also show neutropenia or neutrophil dysfunction and susceptibility to infections whereas these symptoms are not found in type Ia patients [reviewed in 49]. This suggests that glucose 6-phosphate transport has a role to play in neutrophils that is different from the supply of glucose 6-phosphate to glucose-6-phosphatase. A third form of glucose-6-phosphatase defect, GSD Ic, has first been described by Nordlie and coworkers [50]; it appears to be due to a deficiency in the Pi transporter (fig. 3).

Characterization of Glucose-6-Phosphatase Components

Attempts have been made to purify the hydrolase, the most successful one resulting in a 700-fold purification of a 35 kDa protein [51]. The cloning of the cDNA came, however, as a result of an entirely different approach, namely from the isolation of a clone that was underexpressed in the liver of mice deficient in glucose-6-phosphatase as a result of radiation mutagenesis [52]. This clone was shown to encode a transmembrane protein with the two C-terminal lysine residues required to maintain protein in the endoplasmic reticulum [53] and displaying glucose-6-phosphatase activity when expressed in Cos cells [52]. The corresponding gene was shown to be mutated in patients with glycogen storage disease type Ia [44, 45]. Furthermore, knockout mice showed the same symptomatology as patients with glycogen storage disease type Ia [54].

Identification of other constituents is less advanced. Nobody succeeded until now to purify the glucose 6-phosphate translocase. This is probably due to the combination of difficulties inherent to the purification of membrane

proteins but also to the fact that the transporter is actually more difficult to assay than commonly realized. Glucose 6-phosphate transport is not energy-driven and therefore cannot be truly concentrative. However, once transported into the lumen of liver microsomes, glucose 6-phosphate is readily hydrolyzed to glucose and Pi, which accumulate to concentrations that can be higher than the medium glucose 6-phosphate concentration [55]. This explains why almost no accumulation of radioactivity occur when glucose-6-phosphatase is absent, as in GSD Ia liver [56] or in glucose-6-phosphatase knockouts [54], or if the hydrolase is inhibited by vanadate [54]. Other strategies have been devised to measure glucose 6-phosphate transport independently of its hydrolysis [57]. Besides the existence of glycogen storage disease type Ib, one of the most compelling evidences in favor of a glucose 6-phosphate transporter has been the discovery of a new family of compounds that inhibit glucose-6-phosphatase activity in intact but not in detergent-treated microsomes [58, 59]. Since these inhibitors do not act on mannose-6-phosphatase activity or on the microsomal inorganic pyrophosphatase activity, they must inhibit the glucose 6-phosphate translocase.

The Pi translocase has been claimed to crossreact with antibodies directed against the mitochondrial Pi translocase, the main argument being that no reaction was observed in microsomes from a patient with glycogen storage disease type Ic [60]. However, it seems quite surprising that the same Pi transporter would be used in mitochondria and in the endoplasmic reticulum, as it would have to face very different environments, particularly with respect to the transmembrane potential and the phospholipid composition of the membrane. Furthermore, one may also wonder how patients with a defect in the Pi translocase (GSD Ic) could survive if their mitochondrial Pi transporter did not work.

A glucose transporter has also been reported to be present in microsomes and to crossreact with antibodies directed against Glut2 [61]. A cDNA clone that was isolated from an expression library with anti-Glut2 antibodies and shown to have regions of complete identity with Glut2 [62] was recently concluded to be a cloning artefact [63]. The fact that Glut2 mouse knockouts are apparently able to normally produce glucose has led Thorens and coworkers to propose that the glucose that is produced by glucose-6-phosphatase can bypass the cytoplasm and be directly transferred to the circulation by a membrane-traffic dependent mechanism [64]. Such a mechanism would have to be extremely 'active': even if one admits that the glucose concentration in the vesicles would be isoosmotic with the intracellular milieu (300 m*M*), which seems quite unlikely, maximal rates of glucose production (about 3 μmol/min/g wet weight) would require the exocytosis of 1% of the cell volume per minute, a rather high figure. There is, however, evidence in favor of glucose transport

in microsomes [65]. The small volume of these structures makes, however, that the accumulated hexose rapidly escapes during washing, which could explain the negative results recently reported by some authors [66].

The difficulty to characterize the glucose-6-phosphatase components is probably one of the reasons why several authors have disputed the glucose-6-phosphate transport model. Another reason is that rapid filtration techniques have shown that the hydrolysis of glucose 6-phosphate does not show a lag, as expected if the glucose-6-phosphate concentration has first to rise in microsomes before hydrolysis of this phosphate ester proceeds at a constant rate [67]. One may, however, wonder if such changes would indeed be detectable. In the experiment reported in figure 3 by Berteloot and coworkers [67], the initial rate of glucose formation at 0.1 m*M* glucose 6-phosphate ($\approx$0.5 nmol/sec/mg protein) and the admitted intramicrosomal water volume ($\approx$1 µl/mg protein) allows one to calculate that the intravesicular glucose 6-phosphate is renewed five times per second, which makes it difficult to identify a lag phase if the first sample is taken 0.5 second after the initiation of the reaction.

Cloning of the Glucose 6-Phosphate Translocase

Apart from the endoplasmic reticulum, two other types of membrane are known to contain glucose 6-phosphate transporters: (1) the internal membrane of bacteria, which are able to metabolize externally added hexose 6-phosphates [68], and (2) the membrane of plant amyloplasts, which have to exchange hexose-phosphates for Pi with the cytoplasm [69]. The bacterial transporter for hexose-phosphates, known as UhpT, belongs to a family of protein comprising also the transporters for glycerol 3-phosphate (GlpT) and for phosphoglycerate (PgtP), as well as UhpC, whose product appears to be a glucose 6-phosphate receptor that controls the expression of UhpT [68]. This family of proteins belongs to the superfamily of transmembrane facilitators with 12 transmembrane helices. They function actually as antiports that exchange phosphate esters against one or two molecules of inorganic phosphate, depending on the pH [70]. The hexose-phosphate transporters of plant amyloplasts belong to a different family, which comprises also the triose-phosphate/Pi exchangers of the chloroplast membrane.

Search in EST (expressed sequence tags) databanks allowed us to find mouse and human homologues of the bacterial hexose-phosphate translocase [71] but no sequence with significant homology with the plastidial hexose-phosphate translocase. Primers derived from one mouse EST sequence were used to PCR-amplify a 400 bp fragment from mouse liver cDNA. This fragment was used to screen a human cDNA library from a bladder tumor. The

longest clone obtained contained 2,040 nucleotides and predicted a 429 residue protein homologous to the bacterial transporters for phosphate esters (25% identity with GlpT and 20% identity with UhpT) and to UhpC (26% identity). The human protein is very hydrophobic and shows the two lysine residues in position -3 and -4 from the carboxy-terminus that serve as a retention signal for the endoplasmic reticulum [53]. Northern blots showed that a major 2.2 kb mRNA was more abundant in liver and in kidney than in other tissues, as expected for a protein involved in gluconeogenesis [71].

The homology with the bacterial transporters, the tissular distribution and the presence of a targeting signal for the endoplasmic reticulum all suggested that the protein could be the glucose 6-phosphate translocase associated with the endoplasmic reticulum. Proof for this came from analysis of the cDNA from two patients with glycogen storage disease type Ib. One of them was homozygous for a mutation that replaces Gly339 by a cysteine. As this glycine is one of the only 30 residues that are strictly conserved in the bacterial organophosphate transporters, its replacement by a cysteine is most likely to perturb protein function and/or folding. The other patient was heterozygous for this mutation as well as for a mutation replacing Glu355 by a stop codon. This premature stop codon not only results in the absence of the last two helices, but also of the retention signal for the endoplasmic reticulum [71]. These results were recently confirmed by Kure et al. [72] who found different mutations in cDNA from Japanese patients.

Is the Same Gene Mutated in GSD Ib and GSD Ic?

In order to analyze additional patients, the human gene encoding the putative glucose 6-phosphate translocase was cloned [73, 74, 74a]. PCR experiments using DNA from a radiation hybrid pannel indicated that the gene is localized on chromosome 11q23 [72, 73]. This chromosomal localization corresponds not only to that of glycogen storage disease type Ib [75], but also to the locus identified for glycogen storage disease type Ic based on a particularly informative Pakistani family with several affected members [76].

The gene was found to comprise 9 exons, one of which (the 7th exon) is not expressed in liver and kidney, but well in brain and heart [73, 74a, 77]. The 7th exon encodes a 22 (human) or 21 (mouse) amino acid insertion between Lys337 and Leu338 of the liver protein. Its function is presently unknown.

The determination of the sequence allowed the preparation of primers that could be used to amplify genomic DNA from patients reported to have GSD Ib or GSD Ic [73, 74]. In our own series, SSCP analysis and sequencing

Table 1. Mutations in the glucose 6-phosphate translocase. Completely conserved residues in the organophosphate transporter family are in bold. The retention signal for the endoplasmic reticulum is underlined. Mutations are shown below the sequence; letters indicate substitutions; asterisks, stop codons; Δ, changes in reading frame and Σ, splice site mutations. Modified from [73]

MAAQGYGYYR	TVIFSAMFG**G** D	**Y**SLY**Y**FN**R**KT C	**F**SFVMPSLVE	EIPLDKDDL**G**	50
FITSSQSAA**Y** R	AIS**K**FVSGVL R	SDQMSARWLF	SSG**L**LLVGLV D	NIFFA**W**SSTV X	100
PVFAALWFLN	GLA**Q**GLGWPP	CGKKVLRKWFE	PSQF**G**TWWAI	LSTSM**N**LA**G**G R	150
LGPILATILA *	QSYSWRSTLA	LSGALCVVVS	FLCLLLIHNE	**P**ADV**GL**RNLD I	200
PMPSEGKKGS	LKKEESTLQEL X	LLSPYL**W**VLS	TGYLVVFGVK	TCCTD**W**GQFF X	250
LIQEKGQSAL	VGSSYMSAL**E**	VGGLVGSIAA	**G**YL**SD**RAMAK	AGLSNYGNP**R** PX	300
HGLLLFMMAG	MTVSMYLFRV MMAG *	TVTSDSPKLW	ILVLGAVF**G**F G	SSYG**P**IALFG * *	350
VIANESAPPN *	LC**G**TSHAIV**G**	LMANV**G**GFLA ΣΣ	GLPFSTIAKH	YSWSTAFWVA	400
EVICAASTAA	FFLLRNIRTK	MGRVSKKAE			429

of the different exons disclosed the presence of 20 mutations (table 1). Eight of these caused the replacement of a completely conserved (3) or a semi-conserved (5) residue; 4 of them corresponded to premature stop codons and 8 were deletions or insertions also leading to the synthesis of truncated proteins. All mutations appeared therefore to be disease-causing [73].

Interestingly, patients diagnosed as GSD Ic from 2 distinct families (one of which is the Pakistani family used for the localization of the type Ic gene) were found to be mutated in the glucose 6-phosphate translocase gene. Two of the three mutations found in the GSD Ic cases were also found in patients diagnosed as GSD Ib. This finding could indicate either (1) that glucose-6-phosphate and Pi are transported by the same transporter; or (2) that the patients were uncorrectly diagnosed as GSD Ic.

In favor of (1), one should remember that the bacterial translocases, UhpT and GlpT are known to exchange a phosphate ester against Pi. However, this argument is not very strong because the same superfamily of transporters is known to contain antiports, uniports and symports. Furthermore, the inhibitors of the glucose 6-phosphate transport appear not to act on Pi transport, since they do not inhibit inorganic pyrophosphate hydrolysis. It is therefore

likely that the patients diagnosed as GSD Ic are actually GSD Ib patients. The assay used to differentiate both defects is difficult to carry out: it requires the use of microsomes isolated from (ideally) fresh liver biopsy material and is based on the measurement of the latency of microsomal inorganic pyrophosphatase. Furthermore those patients showed neutropenia/neutrophil dysfunction, which appears to be a hallmark of GSD Ib.

Conclusion

The two opposite reactions of the glucose/glucose 6-phosphate cycle are amazingly complex, each of them involving at least two different proteins and different intracellular compartments. In both cases, the *raison d'être* of this compartmentalization still escapes our understanding. Further work may disclose other roles played by the regulatory protein or by glucokinase in the nucleus, which could allow us to provide a satisfactory explanation about the advantage conferred by the translocation phenomemon.

The same is true for glucose-6-phosphatase. Why such a complicated system when it seems so easy to make specific phosphatases such as fructose-1,6-bisphosphatase, L-3-phosphoserine phosphatase etc.? The reason may be in the (marvelous) tinkering that appears to be responsible for evolution. If the glucose 6-phosphate translocase was already well expressed in ancestors of metazoa, one easy way of making a specific hydrolase was to put a nonspecific phosphatase in the compartment to which the translocase gave access. Further characterization of the glucose-6-phosphatase components may one day provide an answer.

In the meantime, the existence of these specific steps gives us interesting targets for the design of drugs useful for the treatment of diabetes and for the understanding of its pathophysiology.

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Emile Van Schaftingen, MD, UCL 7539, Avenue Hippocrate 75, B–1200 Brussels (Belgium)
Tel. +32 2 7647564, Fax +32 2 7647598, E-Mail vanschaftingen@bchm.ucl.ac.be

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Hepatic Glycogen Metabolism in Type 1 Diabetic and Glucokinase-Deficient Subjects

Kitt Falk Petersen, Gerald I. Shulman

Department of Internal Medicine and the Howard Hughes Medical Institute,
Yale University School of Medicine, New Haven, Conn., USA

Recent developments in ^{13}C nuclear magnetic resonance (NMR) spectroscopy have made it possible to measure noninvasively hepatic glycogen content in humans [1, 2]. In this article we will review some recent applications of this technique to examine the regulation of hepatic glycogen synthesis in normal individuals as well as the alterations in hepatic glycogen metabolism that occur in patients with type 1 diabetes and glucokinase deficiency (MODY2).

Basic Principles of NMR Spectroscopy

All atomic nuclei have a positive electrical charge, but only some nuclei also possess magnetic properties the magnetic moment or 'spin'. The magnetic moments of those nuclei are usually randomly orientated in the weak magnetic field of the earth [0.5 G(auss) or 50 μT(esla)]. The presence of a strong static magnet field, however, creates torques on the nuclei causing them to precess around their own axis with a characteristic frequency in order to align with or against the magnetic field. When an oscillating magnetic field (radiofrequency (rf) field) at the precessing frequency of the nuclei is produced by pulses of electromagnetic waves from the rf coil (probe) at the surface over the tissue of interest, energy is transferred to these nuclei causing them to change the angle of precession and swing out of their alignment by 90°. During the interval between the excitation waves the nuclei swing back into their original alignment, which represents a lower energetic level, and thereby emit energy in the form of electromagnetic energy. The signal or 'resonance' is

detected by receiver coils (sometimes the rf coil serves for both excitation and detection of radiowaves) and subject to signal analysis. Under experimental conditions, resonant waves from various nuclei are superimposed giving the picture of oscillating amplitudes in a display of intensities versus time.

To separate the different radiofrequencies, it can be translated into a display of peak intensities vs. frequencies by using a mathematical analysis known as a Fourier transformation. The frequency of a peak is characteristic for a certain nucleus/compound and the area under that peak corresponds to the concentration of that nucleus/compound, which can be converted into molar concentrations by comparison with the area under the peak of a phantom containing a known concentration of that nucleus/compound. The ability to distinguish between different molecules containing the same nucleus relies on the 'chemical shift' (given in parts per million, ppm). The chemical shift can be explained by shielding effects of electrons surrounding the nucleus, which depend on the position of the nucleus within a certain molecule. The nuclei of different molecules thereby experience a gradually altered static magnetic field and in turn resonate at a gradually altered frequency (i.e. chemical shift), which is typical for the respective molecule. The sensitivity of the NMR technique is proportional to the field strength of the magnet used to acquire the spectra and studies in humans are now usually performed at 1.5 T–4.7 T. Moreover, the signal to noise ratio of the spectrum can be enhanced by increasing the total number of rf pulses acquired prior to the Fourier transformation.

In order to examine defined small volumes of tissue, special surface coils made of loops of wire are placed tightly over the region of interest to ensure homogenous tissue filling in that region. The surface coil can also serve for imaging of the region of interest before starting the spectroscopic experiment. The volume selected by a circular surface coil has the shape of an half-sphere. The radius of that disk is the radius of the surface coil and the depth of the volume can be chosen by variation of the pulse power. The pulse angle can be selected so that signals from other tissues like the subcutaneous fat layer are largely suppressed. Because of its low natural abundance (1.1%) ^{13}C has an overall sensitivity of 0.016 compared to 1.00 for ^{1}H. However, even with this low overall sensitivity natural abundance ^{13}C liver and muscle glycogen can be measured in humans due to their relatively high concentrations ranging typically from 50 to 500 mmol/l in liver and 60–140 mmol/l in skeletal muscle. Molar concentrations are estimated by the peak intensity/integral of the C1 glycosidic carbon signal in a proton-decoupled ^{13}C NMR spectrum at 100.5 ppm compared to its intensity in a spectrum obtained from a glycogen phantom. The measurement of tissue glycogen by NMR spectroscopy is based on the observation that all ^{13}C signals from hepatic and skeletal muscle glycogen can be detected by ^{13}C NMR spec-

troscopy [3–6]. ^{13}C NMR spectroscopy can be used to determine natural abundance ^{13}C glycogen [7] but also to measure ^{13}C incorporation into glycogen during infusion or ingestion of [1-^{13}C] enriched glucose (10–99%), which increases the sensitivity of the method and allows one to trace different metabolic pathways by using stable isotope techniques [8–10].

The use of ^{13}C NMR spectroscopy to measure hepatic glycogen content has been validated in liver in vivo by comparison with biopsies in rabbits [2]. These studies demonstrated an excellent agreement ($r = 0.95$) between classical biochemical and NMR spectroscopic measurements of glycogen over a broad range of concentrations. Moreover, ^{13}C NMR spectroscopy was proven to allow precise measurements of glycogen with coefficients of variation between multiple measurements in the same subject of 4.3% (3.4 mmol/l for basal concentrations) in muscle [11] and 7% [1] in liver compared with 9.3% in muscle using the biopsy technique [11].

Hepatic Glycogen Synthesis during Mixed Meal Ingestion

When hepatic glycogen concentrations were measured with ^{13}C NMR spectroscopy in healthy subjects following an overnight fast the mean glycogen concentration was 274 ± 11 mmol/l liver [12]. Following each mixed meal ingestion plasma glucose and insulin concentrations peaked at ~ 30 and ~ 60 minutes, respectively (fig. 1) and liver glycogen content increased continuously throughout the day without any detectable decrease in concentration following breakfast or lunch (fig. 2). Hepatic glycogen concentration peaked approximately four hours following dinner resulting in a net increment of 144 ± 14 mmol/l liver above breakfast concentration (fig. 2). These data demonstrate that during the waking hours of a day, in which three equicaloric mixed meals are ingested at five hour intervals, there is no detectable net hepatic glycogenolysis in contrast to the night (11 pm–7 am) when glycogenolysis contributes $\sim 50\%$ to whole body glucose production [13]. These data suggest that under these conditions most of the glucose demands of the body are met by carbohydrate absorption in the meal and possibly gluconeogenesis.

Hepatic glycogen synthesis occurs by two distinct metabolic pathways, namely, a direct (glucose → glucose-6-phosphate → glucose-1-phosphate → UDP-glucose → glycogen) or indirect (gluconeogenic) (3-carbon units → → phosphoenolpyruvate → → glucose-6-phosphate → glucose-1-phosphate → UDP-glucose → glycogen) pathways [14–15]. In order to assess the relative contribution of these pathways to hepatic glycogen synthesis we added [1-^{13}C]glucose to an identical breakfast meal on the following day along with acetaminophen to noninvasively sample the hepatic UDP-glucose pool [16–18].

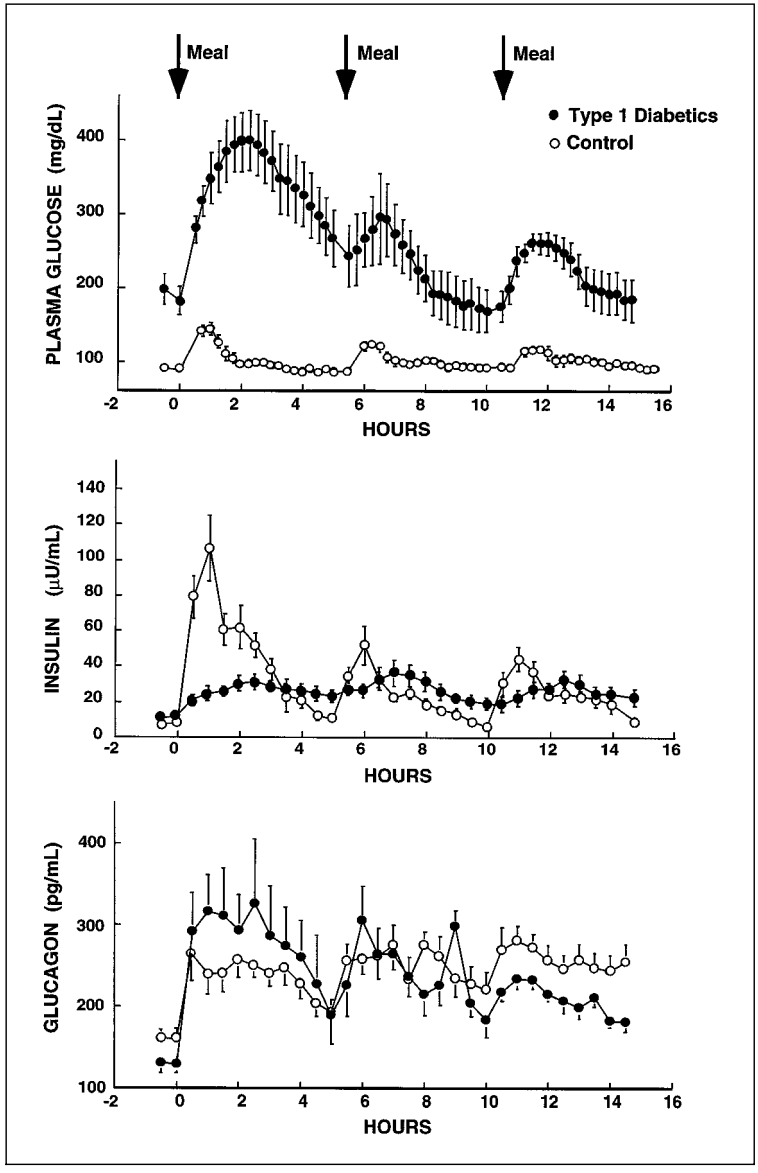

Fig. 1. Plasma glucose, insulin and glucagon concentrations in normal ○ and type 1 diabetic subjects ● following mixed meal ingestion at: 7:00 am, 1:00 pm and 6:00 pm.

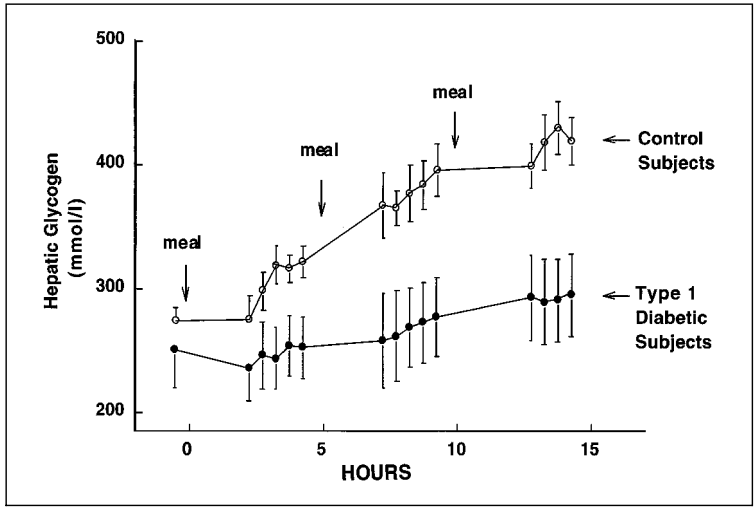

Fig. 2. Hepatic glycogen concentration in normal ○ and type 1 diabetic subjects ● following mixed meal ingestion at: 7:00 am, 1:00 pm and 6:00 pm.

Using this approach we found that the mean relative flux of the direct pathway for hepatic glycogen synthesis following breakfast was ~55% and the relative flux through the direct pathway increased during the day such that by the last time interval (4 to 5 hours after breakfast), 71 ± 10% of hepatic glycogen was synthesized by the direct pathway (fig. 3).

Effect of Type 1 Diabetes

When poorly controlled type 1 diabetic subjects were studied under the same protocol both fasting and postprandial plasma glucose concentrations were markedly higher at all time points due to insulinopenia (fig. 1) [12]. Although there were no differences in the mean plasma glucagon concentrations between the type 1 diabetic and the healthy control subjects at any time, the mean plasma glucagon concentrations were inappropriately elevated in the type 1 diabetic subjects considering their level of hyperglycemia. The concentration of plasma C-peptide measured before and after each meal was undetectable in all of the type 1 diabetic subjects.

The time course for mean hepatic glycogen concentration in comparison to the healthy subjects is shown in figure 2. The mean fasting hepatic glycogen concentration was slightly, but not significantly, lower in the diabetic subjects

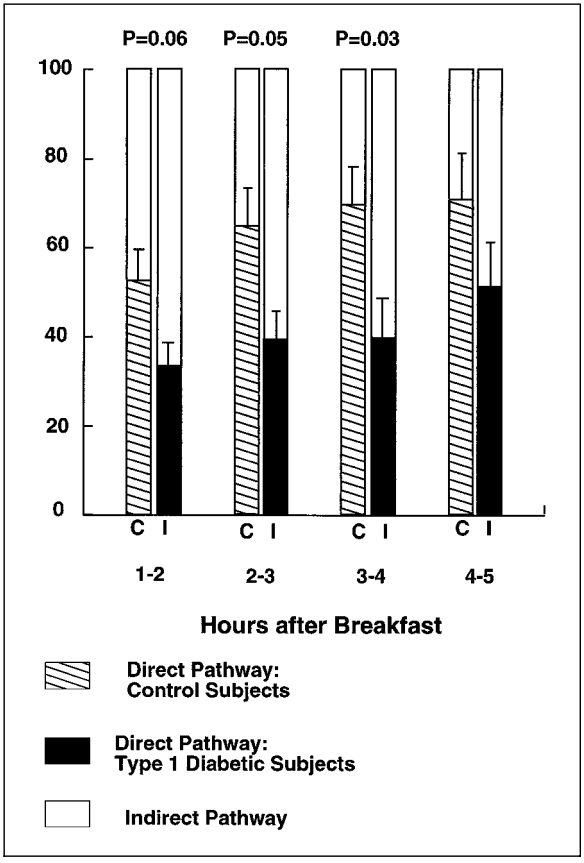

Fig. 3. Relative contributions of the direct (%) and indirect (%) pathways to hepatic glycogen synthesis in normal and type 1 diabetic subjects following mixed meal ingestion at: 7:00 am.

(250 $\pm$ 30 mmol/l liver) than in the control subjects (274 $\pm$ 11 mmol/l liver). While the overall pattern of net hepatic glycogen synthesis was similar in the type 1 diabetic and the control subjects, the net amount of hepatic glycogen synthesized by the type 1 diabetic subjects was significantly less than in the control subjects. At 11 pm (four hours following dinner) hepatic glycogen stores were at their maximum concentration in both groups but the type 1 diabetic subjects had synthesized only $\sim$30% of the hepatic glycogen that was made by the control subjects.

Figure 3 shows the mean relative flux of the direct pathway for hepatic glycogen synthesis following breakfast as assessed by the acetaminophen-

glucuronide method. During the first four hours following breakfast the relative flux through the direct pathway of hepatic glycogen synthesis was between 1.6 to 1.8 fold greater in the control subjects compared to the type 1 diabetic subjects. The relative flux through the direct pathway increased in both groups such that by the last interval (4 to 5 hours), $71 \pm 10\%$ and $51 \pm 10\%$ ($p = 0.03$) of hepatic glycogen was synthesized by the direct pathway in the control and type 1 diabetic groups, respectively. This data demonstrates that hepatic gluconeogenesis is increased in the poorly controlled type 1 diabetic individuals and what little hepatic glycogen they synthesized was made mostly by the gluconeogenic pathway.

When portal insulin and glucagon concentrations were matched to those of nondiabetic individuals during a hyperglycemic-hyperinsulinemic clamp, rates of hepatic glycogen synthesis were normalized (0.49 ± 0.10 μmol/ml liver·min in the control group and 0.46 ± 0.07 μmol/ml liver·min in the type 1 diabetic subjects) [19]. In contrast, matching portal vein concentrations of glucose, insulin, and glucagon did not normalize the relative fluxes of hepatic glycogen synthesis and the indirect pathway was found to be about twice as active in the type 1 diabetic subjects as in the healthy control subjects (0.15 ± 0.02 and 0.28 ± 0.06 μmol/ml liver·min; $p < 0.05$). These results are also in accord with studies in normal and partially pancreatectomized rats where no differences in net hepatic glycogen synthesis rates were found but where there was a significant increase in the indirect pathway [20].

In summary, these studies demonstrate that poorly controlled type 1 diabetic individuals have a profound defect in net hepatic glycogen synthesis and that decreased hepatic glycogen reserves in the later part of the day may be an important factor contributing to the impaired glycemic response to counterregulatory hormones in type 1 diabetic subjects. Furthermore augmented gluconeogenesis may be an important contributing factor to postprandial hyperglycemia. The degree to which these abnormalities can be reversed with more aggressive insulin therapy remains to be determined.

Effect of Glucokinase Deficiency (MODY2)

In order to examine what impact hepatic glucokinase deficiency has on net hepatic glycogen synthesis we applied the same mixed meal feeding protocol to six well controlled patients with glucokinase deficiency [21]. These subjects had normal glycosylated hemoglobin and were all in good health.

Fasting plasma glucose concentrations were slightly, but significantly, higher in glucokinase-deficient subjects than in control subjects, and remained significantly higher throughout the day (fig. 4). Fasting plasma insulin was

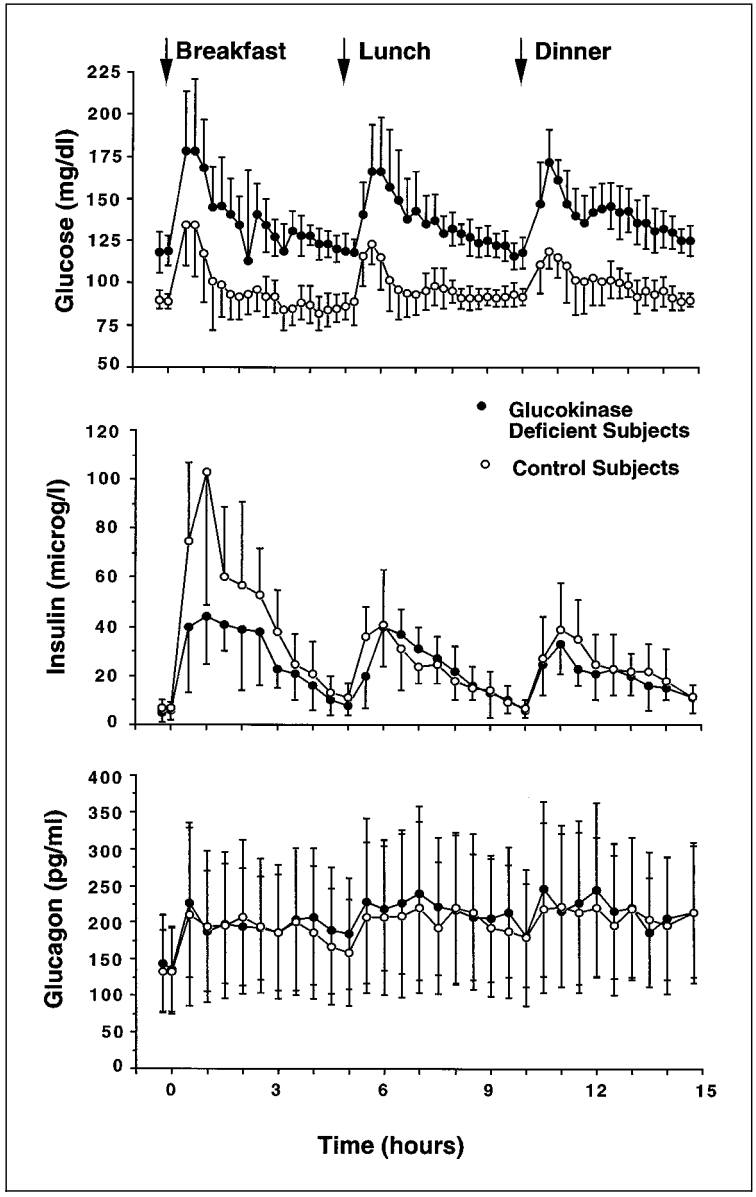

Fig. 4. Plasma glucose, insulin and glucagon concentrations in normal and glucokinase-deficient (MODY2) subjects following mixed meal ingestion at: 7:00 am, 1:00 pm and 6:00 pm.

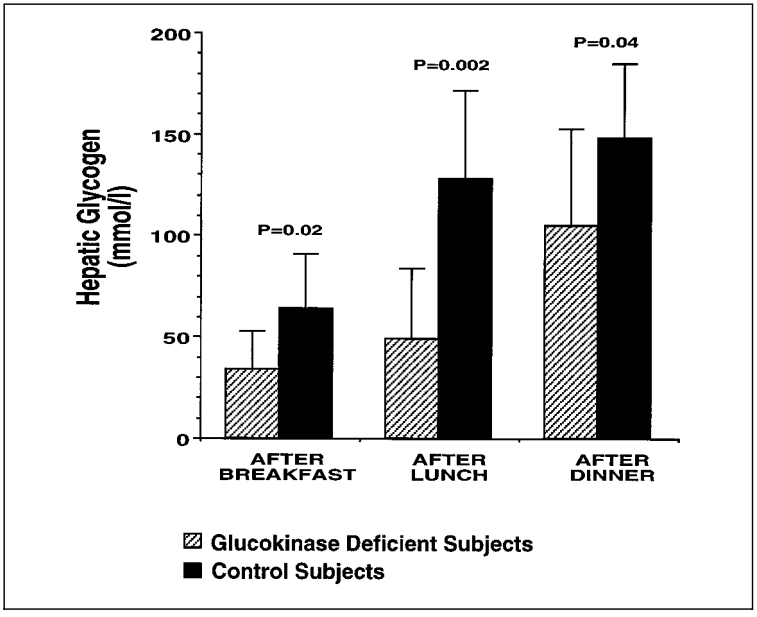

Fig. 5. Incremental changes in net hepatic glycogen synthesis in normal and glucokinase-deficient (MODY2) subjects following mixed meal ingestion at: 7:00 am, 1:00 pm and 6:00 pm.

not significantly different between the two groups. One hour after the ingestion of breakfast, plasma insulin peaked at 264 ± 43 pmol/l in patients and 618 ± 99 pmol/l in controls ($p < 0.03$). The overall insulin secretory response as expressed by the area under the insulin curve from 0–5 hours after the breakfast was significantly reduced in the MODY subjects [$48{,}690 \pm 6{,}674$ pmol/l-min vs. $79{,}494 \pm 7{,}261$ pmol/l-min in the control subjects ($p = 0.01$)]; but three and a half hours after breakfast plasma insulin concentration was again similar in glucokinase-deficient and control subjects and remained similar throughout the rest of the day. Following lunch as well as dinner the overall insulin secretory responses were similar in the MODY and the control subjects. Similar profiles of plasma glucagon were observed in both groups throughout the day.

Fasting hepatic glycogen concentrations were similar in glucokinase-deficient and control subjects (279 ± 20 vs. 284 ± 14 mmol/l). Hepatic glycogen concentration increased in a similar pattern in both groups throughout the day. However, the mean increment in hepatic glycogen content following each meal was typically 30–60% lower in the glucokinase-deficient subjects compared to the control subjects [breakfast; 46% lower – $p < 0.02$, lunch; 62% lower – $p < 0.002$, dinner; 30% lower – $p = 0.04$ (fig. 5)]. Peak hepatic glycogen

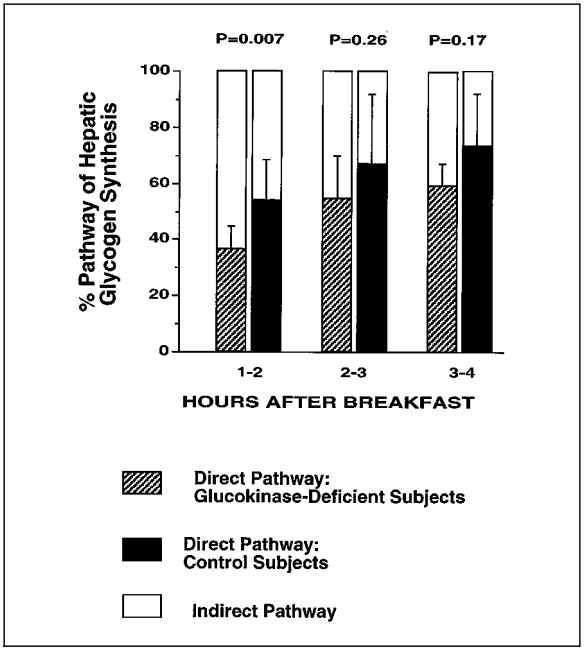

Fig. 6. Relative contributions (%) of the direct and indirect pathways to hepatic glycogen synthesis in normal and glucokinase-deficient (MODY2) subjects following mixed meal ingestion at: 7:00 am, 1:00 pm and 6:00 pm.

concentration four hours after breakfast tended to be lower in the glucokinase deficient subjects (313 ± 20 vs. 347 ± 14 mmol/l ($p = 0.17$)) and was significantly decreased after lunch and dinner (333 ± 22 vs. 414 ± 17 mmol/l ($p = 0.02$), and 383 ± 16 vs. 434 ± 14 mmol/l ($p = 0.04$), in glucokinase-deficient and control subjects, respectively).

The mean relative flux of the direct pathway for hepatic glycogen synthesis after breakfast for each group is shown in fig. 6. During the second hour following breakfast (1–2 h interval) the relative flux through the direct pathway was significantly less in the glucokinase-deficient subjects than in controls (36 ± 3 vs. $55 \pm 4\%$; $p = 0.007$). Flux through the direct pathway increased in both groups such that by the last interval (3–4 h), $60 \pm 3\%$ and $74 \pm 8\%$ of hepatic glycogen was synthesized by the direct pathway in glucokinase-deficient and control subjects, respectively.

The results of these studies show that glucokinase-deficient subjects have a decreased accumulation of hepatic glycogen throughout the day as compared to control subjects. This defect was observed not only after breakfast, when

glucokinase-deficient subjects presented with reduced plasma insulin concentrations, but also following lunch and dinner in the presence of similar plasma insulin and glucagon concentrations. This observation suggests that the defect in hepatic glycogen synthesis is indeed related to the decreased enzymatic activity of mutant GCK, and not to an acquired alteration of hepatic glucose metabolism, as we have observed in subjects with poorly controlled insulin dependent diabetes mellitus [12]. Five hours after dinner, the increment over basal values of net glycogen concentration in glucokinase-deficient subjects was 70% of the increment observed in the control subjects. Considering that the glucokinase-deficient subjects had significantly higher plasma glucose concentrations throughout the day compared to the control subjects implies an even lower efficiency of hepatic glycogen synthesis in terms of glucose availability than the control subjects.

The gluconeogenic pathway was relatively more important for synthesizing hepatic glycogen in the glucokinase-deficient subjects. In the first 2 hours following breakfast more than 60% of the glycogen was derived from gluconeogenesis in the glucokinase-deficient subjects compared to less than 50% in the control subjects. The percentage of hepatic glycogen synthesized by the direct pathway increased in both groups in the third and fourth hours after breakfast, but remained lower in glucokinase-deficient subjects than in controls, although not significantly different. The mechanisms controlling the contributions of the direct and the indirect pathways to hepatic glycogen synthesis are not fully understood, but the preferential utilization of the gluconeogenic pathway may result at least in part from the lower insulin/glucagon ratio observed in glucokinase-deficient subjects after breakfast [9]. Whatever the mechanism responsible for the increased flux through the gluconeogenic pathway, this effect partially explains the mildness of the defect of net hepatic glycogen synthesis observed in these subjects. It is also possible that increased hepatic glycogen cycling may also be partly responsible for the lower rates of net hepatic glycogen synthesis in the glucokinase-deficient subjects.

In recent studies we have observed that hepatic glycogen synthase and glycogen phosphorylase can be simultaneously active in humans under conditions of net glycogen synthesis resulting in glycogen cycling [8–10]. The consequences of these GCK mutations on hepatic glycogen turnover remain to be determined. Normal glucose tolerance after a meal also depends on normal suppression of hepatic glucose production [22]. In this regard increased relative rates of gluconeogenesis observed following a meal in the glucokinase-deficient subjects while probably beneficial in terms of hepatic glycogen repletion, is also likely an important contributing factor to postprandial hyperglycemia in these subjects. In support of this, Clement et al. [23] have observed abnormal suppression of hepatic glucose production by physiological levels of insulin

(252 ± 27 pmol/l) during a euglycemic clamp in a small number of glucokinase-deficient subjects. Taken together, these observations underscore the key role of the liver in the pathophysiology of hyperglycemia associated with glucokinase deficiency.

In conclusion, mutations in the coding regions of GCK are associated with decreased net accumulation of hepatic glycogen and augmented hepatic gluconeogenesis following meals. The former abnormality probably results from impaired glucose phosphorylation in hepatocytes due to decreased enzymatic activity of mutant glucokinase. Both abnormalities may contribute to postprandial hyperglycemia. These results indicate that the hyperglycemia of glucokinase-deficient type 2 diabetes results from primary abnormalities of glucose metabolism in both the pancreatic β-cells and the liver.

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Kitt Falk Petersen, MD, Yale University, School of Medicine,
Fitkin 104, PO Box 208020, New Haven, CT 06520-8020 (USA)
Tel. +1 203 737 1942, Fax +1 203 785 6015, E-Mail kitt.petersen@yale.edu

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Role of PDX-1 in Pancreatic Development

Maureen Gannon[a], *Kevin Gerrish*[b], *Roland Stein*[b],
Christopher V.E. Wright[a]

[a] Department of Cell Biology and
[b] Department of Molecular Physiology and Biophysics, Vanderbilt University Medical Center, Nashville, Tenn., USA

Pancreas Development

The development of the pancreas provides an attractive system in which to study the processes of region-specific endodermal development and cell lineage diversification from a simple epithelium, and how these processes produce an organ capable of sophisticated integrated functions associated with mammalian homeostasis. In addition, understanding pancreatic development at the molecular level has strong implications for understanding the etiology of diabetes and the design of new therapies. The restricted expression pattern of the PDX-1 homeodomain protein in the posterior foregut and throughout pancreatic outgrowth and differentiation, as well as in vitro evidence that it is a potent insulin gene transactivator [1–3], identify this factor as a key regulator of organogenesis and mature cell function of this region of the endoderm. Importantly, *pdx-1* null mutations in both mouse and human lead to pancreatic agenesis [5–7]. Moreover, heterozygous mutant mice show symptoms of glucose intolerance, and heterozygous humans show increased susceptibility to mature onset diabetes [8, 9]. Indeed, the recent identification of *pdx-1* as the MODY4 gene [10] highlights its importance in pancreatic development and islet cell function throughout life. Here, we review recent data on the role of *pdx-1* in pancreas development, and the requirement for PDX-1 in adult islets for proper glucose homeostasis. We will also briefly describe strategies being taken by our laboratory to understand how *pdx-1* expression is regulated in the posterior foregut and in islet endocrine cells.

The advent of molecular genetic analysis of embryogenesis has also led to a surge of information about the factors regulating pancreatic growth and differentiation, and islet cell-specific gene expression in particular. In mouse embryos, the pancreas arises from dorsal and ventral evaginations of the posterior foregut endoderm (the smaller ventral pancreatic bud is derived from an outgrowth just posterior to the developing liver), which begin to form at 9.0 d.p.c. By mid-gestation, both outgrowths fuse to form a single organ. There is fairly strong evidence that the dorsal and ventral pancreatic buds can be considered to arise through independent mechanisms [6, 11–14]. For example, mouse embryos homozygous for a null mutation of the *Isl1* gene, which encodes a LIM-homeodomain transcription factor, do not form a dorsal pancreatic bud. This phenotype is currently thought to indicate that *Isl-1* expression in the dorsal lateral mesoderm is required to initiate a signaling function directed at the endoderm to induce bud outgrowth. In contrast, these embryos do form a ventral pancreatic bud containing differentiated acinar cells, but the complete absence of islets within it demonstrates the essential nature of *Isl-1* for pancreatic endocrine cell development [11].

There is much experimental evidence that both the endocrine (islet) and exocrine (acinar) cells arise from common precursor cells located within the ductal epithelium of the pancreas [6, 15, 16]. Both acinar and endocrine precursors are presumed to bud from the distal end of the growing ducts [17–19]. Almost nothing is known about the mechanisms that bias ductal cells toward an acinar or endocrine fate, although they are likely to include intercellular signals, as well as interactions with the extracellular matrix. Transcription of endocrine-specific hormone genes (e.g. insulin, glucagon) in the pancreatic buds is first detected at 9.5 d.p.c. prior to formation of a ductal epithelium or islet morphogenesis [20, 21]. Differentiating endocrine cells are initially found in close proximity to the ducts throughout embryogenesis (beginning in $\sim$12 d.p.c. embryos), but then begin to become organized into clusters that are nonclonal in origin, becoming separated from the ducts toward the end of gestation ($\sim$18.5 d.p.c.) [22, 23].

There is evidence that endocrine cells initially coexpress multiple hormones, and become restricted in their potential during embryogenesis, until they eventually express a single hormone associated with the mature α (glucagon), β (insulin), or δ (somatostatin)-secreting endocrine fate [21]. Current models for endocrine cell lineage pathways may, however, have to be modified in the future since they have so far been inferred from the embryonic stages at which the various cell types appear. This situation is likely to be remedied with the development of lineage-tracing techniques that will allow the gathering of definitive experimental evidence for particular steps in these pathways. Knowledge of factors regulating β-cell production and function, and insulin

production, will have enormous relevance to understanding the development of diabetes, and may eventually lead to therapies that directly stimulate β-cell production and/or improve the function of existing β-cells.

pdx-1 Expression during Pancreas Development

In all vertebrates examined to date, progenitors of the rostral duodenum and pancreas share expression of *pdx-1* [6, 12, 15, 24–26], a gene first isolated from *Xenopus* (initially called *XlHbox8*; [24]. The mammalian homologue was identified independently in several labs based on its expression in islet endocrine cell types, and its ability to transactivate insulin or somatostatin transcription through specific upstream DNA sequences found in these genes [25, 27, 28]. Our analysis of *pdx-1* expression in the mouse was enhanced by the introduction of a *lacZ* reporter gene into the locus via homologous recombination in ES cells [6]. This produced a *pdx-1* allele ($pdx\text{-}1^{lacZKO}$) in which β-galactosidase expression is controlled by endogenous *pdx-1* regulatory elements. Characterization of X-gal staining patterns showed that *pdx-1* expression precedes insulin and glucagon, beginning at 8.0 d.p.c. in a small patch of ventral foregut endoderm adjacent to the anterior intestinal portal and laterally contiguous with the extraembryonic membranes (fig. 1). Dorsal bud *pdx-1* expression begins approximately one day later. During the stages when *pdx-1* expression first becomes activated, the anterior-ventral surface of the foregut endoderm contacts cardiac mesoderm, while the dorsal foregut lies very close to the notochord, an axial mesodermal derivative. Studies in chick embryos have shown that signals from the notochord induce *pdx-1* expression in the dorsal pancreatic bud, and its outgrowth and differentiation [12]. The spatiotemporally distinct expression of *pdx-1* in the ventral bud highlights the possibility of separate dorsal and ventral bud-inducing signals, in keeping with the $Isl\text{-}1^{-/-}$ mutant phenotype described above. The signals involved in ventral bud formation might emanate from the cardiac mesoderm or adjacent extraembryonic tissues or, alternatively, they may arise in an endoderm-autonomous manner as a result of region-specific differentiation programs established at very early stages of embryogenesis. In adults, *pdx-1* expression is maintained in rostral duodenal cells and islet β-cells, and at lower levels in subsets of acinar and somatostatin-producing cells [15, 26, 29]. The expression pattern alone suggests that PDX-1 plays multiple roles during embryogenesis and in the adult, including specification of the posterior foregut, proliferation and differentiation of different pancreatic lineages, activation of insulin gene expression, and/or maintenance of islet and β-cell functions related to glucose homeostasis.

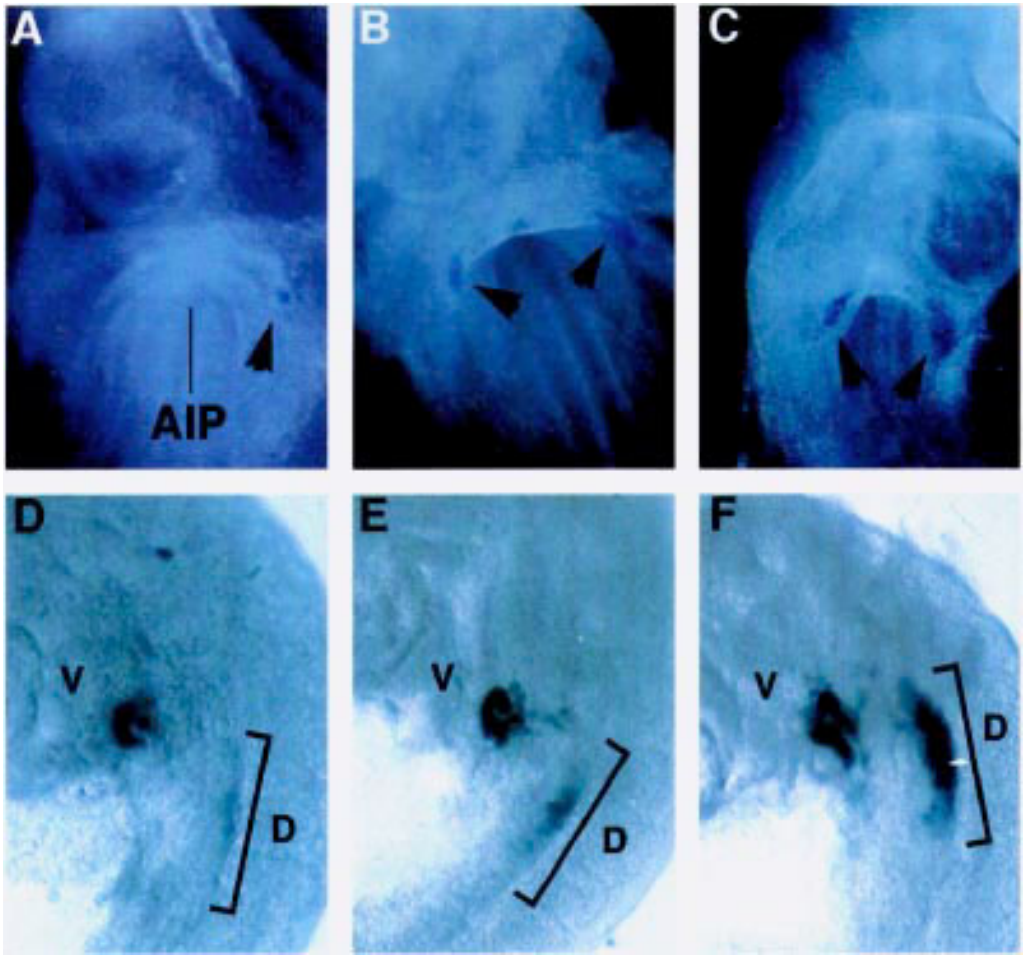

Fig. 1. pdx-1^{lacZ} expression during early embryogenesis. Ventral *A–C* or lateral *D–F* views of mouse embryos in temporal sequence from 8.0 d.p.c. to 9.5 d.p.c. β-galactosidase (β-gal) expression (dark blue) marks *pdx-1* expressing cells in the posterior foregut endoderm. *A pdx-1* is first detected at ∼8.0 d.p.c. (7 somites) on the left ventral side of the AIP (arrowhead). *B* By the 8- to 9-somite stage, expression is observed on both left and right sides ventrolateral to the AIP (arrowheads). *C–F* Between 9.0–9.5 d.p.c., the ventral expression domains fuse as the ventral foregut closes anteriorly. *D* Dorsal bud expression of *pdx-1* is first detected at 9.0 d.p.c. (bracket). This expression expands *E–F* and intensifies until it becomes the predominant bud. AIP = Anterior intestinal portal; D = dorsal; V = ventral; d.p.c., days post-coitum. Anterior is toward the top in *A–C*, and the upper left in *D–F*. [13].

Summary of *pdx-1* Mutant Phenotype

The mouse *pdx-1* gene has been inactivated through targeted mutagenesis in ES cells. Newborn *pdx-1* homozygous null mice lack a pancreas and die a few days after birth [5, 6]. The relevance of these findings is underscored by the identification of a truncating *pdx-1* mutation in humans, leading to the production of a dominant negative protein that retains DNA binding activity but cannot activate transcription [30]. This *pdx-1* mutation also causes pancreatic agenesis in homozygous humans [7]. Our analysis of *pdx-1*^{lacZ} mouse embryos allowed *pdx-1* expressing cells to be identified easily in heterozygous and homozygous null animals, providing further insight into the role of *pdx-1* in pancreatic specification and differentiation. It is now clear that pancreas development in *pdx-1* null mice initiates normally at 9.5 d.p.c., but that only the very first stages of pancreatic outgrowth occur (fig. 2) [6]. By 11.5 d.p.c., the ventral bud is no longer detectable, perhaps because its derivatives somehow become incorporated into the rostral duodenal epithelium [6]. The dorsal bud forms a minimally branched epithelial ductule, which essentially remains in this state throughout embryogenesis into newborn stages. Mature acinar or islet cells do not form adjacent to the partially outgrown ductular epithelium, although transient populations of insulin-positive cells [16], and longer-lived glucagon-positive cells, are observed. Paradoxically, despite its isolation as a putative insulin gene transactivator, the detection of insulin-positive cells in homozygous *pdx-1* null mice shows that at least in these early cells, insulin is expressed in the absence of PDX-1. It is currently unclear, however, whether these early *pdx-1*-independent endocrine cells, which have different characteristics from islet β-cells, are lineally related to mature islets, or if the mature islet cells are all derived from a separate, *pdx-1*-dependent, cell population. Again, the establishment of cell fate-independent lineage-tracing techniques that can be applied to the developing endoderm and pancreas may help to address these questions.

Other cell types of the posterior foregut that normally express *pdx-1* are also affected in the homozygous null mutant mice. The pylorus and rostral duodenum are greatly malformed, and the Brunner's glands at the neck of the rostral duodenum are absent [6]. In addition, the numbers of enteroendocrine cells expressing serotonin, secretin and CCK in the rostral duodenal mucosa are greatly reduced, and there is a substantial alteration in the number and type of secretory cells in the antral stomach [6, 31].

In summary, the activation of *pdx-1*^{lacZ} expression, formation of pancreatic buds and the limited pancreatic outgrowth in *pdx-1* mutants, in the absence of proper terminal differentiation, shows that initial pancreatic specification is independent of *pdx-1*, and that other factors/genes control the regional

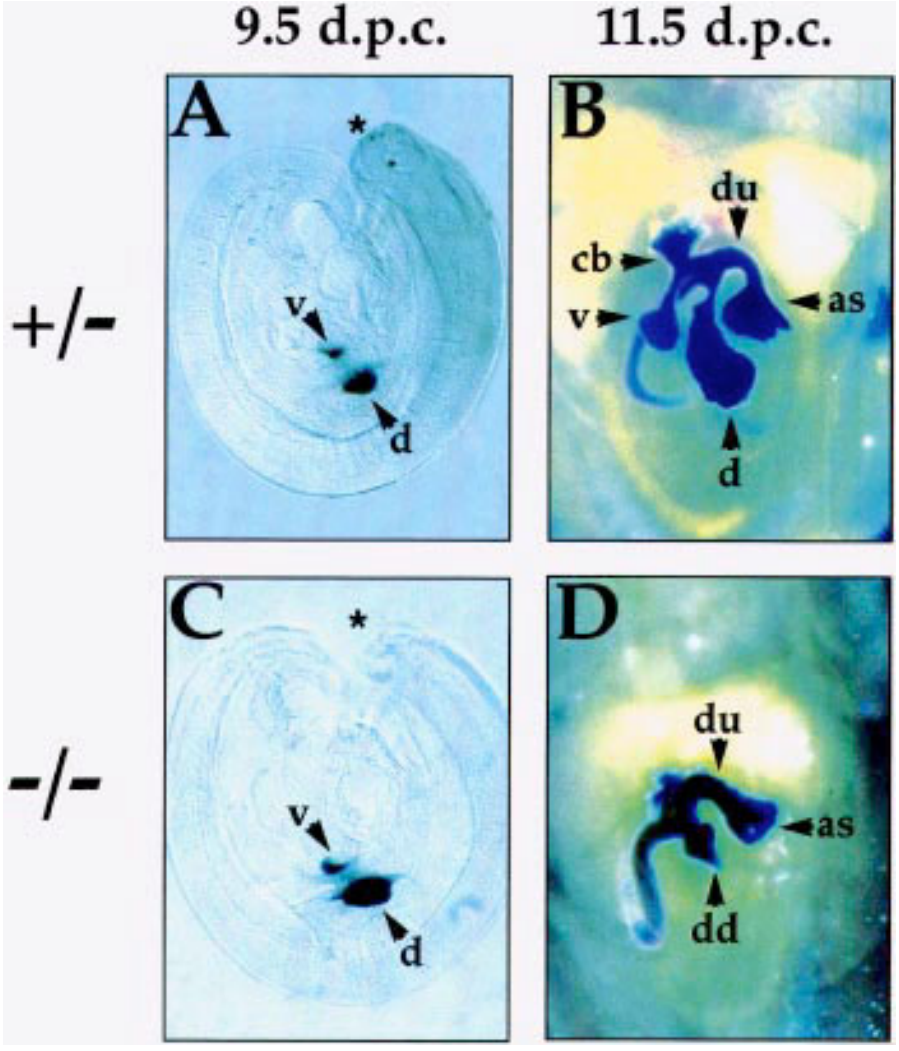

Fig. 2. Pancreatic bud development in PDX-1 (+/–) and (–/–) embryos visualized via β-gal expression from the *pdx-1*^{lacZ} 'knock-in' allele. *A, C* At 9.5 d.p.c., *pdx-1* expression (blue) can be seen in the developing dorsal and ventral pancreatic buds, as well as in the rostral duodenum at lower levels, in both wild-type and mutant animals. Anterior is at upper right (asterisk); heads were removed for genotyping. *B, D* Developing posterior foregut of (+/–) and (–/–) animals at 11.5 d.p.c. *B* By this stage, the dorsal and ventral buds have grown in (+/–) embryos and *pdx-1* is expressed throughout the pancreatic buds, rostral duodenum, antral stomach and common bile duct. *D* In contrast, pancreatic buds are absent in (–/–) embryos and a unique ductal structure (dd) replaces the dorsal bud. The pattern of *pdx-1* expression is otherwise identical to (+/–) embryos. d = Dorsal pancreatic bud; v = ventral pancreatic bud; as = antral stomach; du = duodenum; dd = dorsal ductule; cb = common bile duct [13].

specification of the pancreas and duodenum from the endoderm. *pdx-1* expression confers the pancreatic epithelium with the competence to respond to noninstructive mesenchymal signals that drive subsequent pancreatic growth and differentiation [16].

Role of *pdx-1* in Adult Islets

The continued expression of *pdx-1* in adult β-cells, and the evidence that PDX-1 can activate transcription of insulin and β-cell genes in vitro support a role for *pdx-1* in mature islets, and implicates PDX-1 dysfunction as a contributing factor in some forms of diabetes (discussed further below). In principle, several different functions could be imagined for PDX-1 in mature islets, particularly in β-cells. For example, PDX-1 might couple the level of insulin expression to stimuli such as increased blood glucose, or directly maintain β-cell function by regulating gene products involved in glucose sensing and transport. Support for this proposal comes from the observation that DNA binding and activation potential of PDX-1 are regulated by physiological glucose concentrations [32, 33]. Strong circumstantial evidence supports the idea that PDX-1 is an important transcriptional activator of genes central β-cell/function. For example, PDX-1 and basic helix-loop-helix (bHLH) transcription factors can synergistically activate insulin promoter/reporter constructs [10, 34–36], and PDX-1 can activate genes like GLUT-2, glucokinase and islet amyloid polypeptide (IAPP) [3, 37–39]. Together with the proven requirement for *pdx-1* during pancreas development, these findings have stimulated enormous interest in precisely defining its function in later aspects of pancreas differentiation and function. Analysis of *pdx-1* (+/–) adult mice led to the finding that their islets were slightly smaller with an increased non-β-cell mass. Moreover, their ability to clear glucose from the bloodstream was severely impaired, indicating that glucose homeostasis is sensitive to *pdx-1* gene dosage [8, 9]. Exactly how a decreased level of PDX-1 translates into decreased glucose responsiveness is currently unclear, but the finding that β-cell expression of the glucose transporter, GLUT2, is reduced in PDX-1 (+/–) adults [9] is consistent with a direct role for PDX-1 in GLUT2 regulation, as also suggested by in vitro gene regulation studies [37]. Even if the reduction in GLUT2 alone is insufficient to explain this phenotype, it is possible that compromised glucose sensing in islets with reduced PDX-1 contributes to the heterozygote glucose intolerance.

The early failure of pancreas development in *pdx-1* (–/–) mutants initially presented a stumbling block to the genetic analysis of later aspects of *pdx-1* function in acinar and islet differentiation, and the specific question of how

pdx-1 expression is linked to insulin gene regulation and/or mature β-cell function. To circumvent this problem, the Cre-*lox* method for conditional gene inactivation was recently employed [9]. This binary system allows control over the timing and cell type in which the gene of interest is inactivated [40, 41]. When mice carrying a rat insulin promoter (RIP)-driven Cre transgene were crossed to mice homozygous for a *pdx-1* allele in which the second exon was flanked by *lox*P sites (a so-called 'floxed' locus), Cre recombinase produced in insulin-expressing cells removed the second exon, thereby inactivating *pdx-1* in a cell-type specific manner. Interestingly, although activity from the RIP-Cre transgene was detected in insulin positive cells from very early embryonic stages onward, the phenotype observed was a late, adult onset type of diabetes beginning at ~3–5 months of age. A possible resolution of this discrepancy is that cells must express a particular threshold level of Cre recombinase to cause efficient inactivation of the floxed *pdx-1* locus, a condition only fulfilled in later insulin-positive cells. A linked possibility, based upon PDX-1 acting as a positive regulator of the insulin gene, is that the level of RIP activity became reduced as the inactivation of *pdx-1* proceeded. Examination of specific markers (transcription factors and a selection of other proteins) revealed that RIP-Cre expression reduced the number of PDX-1 expressing cells by 80% by 18 weeks of age. At the same time, the number of islet cells containing either insulin or IAPP was reduced by 40%, and the β-cell-specific homeodomain protein Nkx6.1 was virtually undetectable. An unexpected observation, however, was a greatly increased number of glucagon-positive cells that were uncharacteristically distributed throughout the islet core rather than occupying their normal peripheral positions. These results support a requirement for *pdx-1* in insulin, IAPP and Nkx6.1 expression. Furthermore, they also suggest that PDX-1 directly or indirectly represses glucagon expression in β-cells. These studies did not distinguish whether loss of *pdx-1* from β-cells induced an 'overall' conversion of β-cells to an α-cell like phenotype, whether the glucagon-positive cells arose from an abnormally expanded α-cell population, or whether glucagon expression was superimposed on cells retaining some β-cell characteristics. In relation to the latter point, immunohistochemical detection of residual long lived proteins, such as insulin, may result in the scoring of some cells as overlapping in phenotype, when in fact, these β-cells may have been transformed into true α-cells upon *pdx-1* inactivation. Nevertheless, these results raise the intriguing idea that expression of *pdx-1* may 'tip the scales' of a bipotential α/β progenitor in favor of the β-cell fate.

In conclusion, the analysis of mice with reduced *pdx-1* gene dosage, or reduced numbers of cells expressing *pdx-1*, suggest that lowering the level of PDX-1 activity could contribute to type 2 diabetes by affecting the levels of insulin and GLUT2.

Transgenic Analysis of *pdx-1* Regulation

The identification of *pdx-1* as a key regulator in the pathway of pancreatic differentiation, coupled with its conserved posterior foregut expression pattern in many different vertebrates, make it not only highly relevant to human disease, but also an ideal entry point for dissecting the mechanisms of endoderm regionalization and islet-specific gene expression. In analyzing how *pdx-1* expression is restricted to the posterior foregut, and what regulates its dynamic pattern of expression throughout development, one important goal is to determine whether separate *cis*-acting regulatory modules control duodenal and pancreatic expression of *pdx-1*. Functional characterization of such elements may provide clues towards the identity of upstream factors that define the pancreatic region of the endoderm. As a putative insulin gene regulator, it will also be useful to identify nucleotide sequences driving *pdx-1* expression in β-cells during bursts of islet cell growth, in mature islets, and whether glucose-responsive elements exist that augment *pdx-1* expression in response to transiently increased demands to maintain glucose homeostasis.

To begin such an analysis in the most efficient manner, we have used the complementary approaches of in vitro analysis in cell lines transiently transfected with chloramphenicol acetyl transferase (CAT) reporter constructs, and in vivo characterization of mice bearing *lac*Z reporter transgenes. By comparing the relative levels of *pdx-1*-driven CAT activity in β- and non-β-cell lines, we have fairly rapidly identified regions of *pdx-1* directing expression specifically β-cells. The in vivo study of these modules in transgenic mouse lines, although much slower, not only allows confirmation of in vitro data in the context of the whole animal, but allows analysis of their activity throughout embryogenesis, postnatal and adult life.

Initially, we found that a 14.5 kb transgene encompassing the *pdx-1* locus was sufficient to rescue islet and acinar differentiation in mice that are homozygous null at the endogenous *pdx-1* locus. Thus, we presume that this clone contains most, if not all, of the key *pdx-1* regulatory elements (M.G. and C.V.E.W., unpubl. observations). Next, a reporter transgene corresponding to this clone was made by inserting a *lac*Z cassette into the second exon. Expression of this reporter in embryos and adults recapitulates the endogenous *pdx-1* expression pattern in the rostral duodenum and pancreas (fig. 3) [29]. Subsequently, it was found that the same expression pattern could be given by approximately 4.5 kb of DNA lying 5′ of the transcription initiation sites, and that intron and 3′ sequences were dispensable (L. Gamer, M. Offield and C.V.E.W., unpubl. observations).

Within this 5′ region, smaller independent modules directing *pdx-1* expression to different subsets of pancreatic cell types are now being defined.

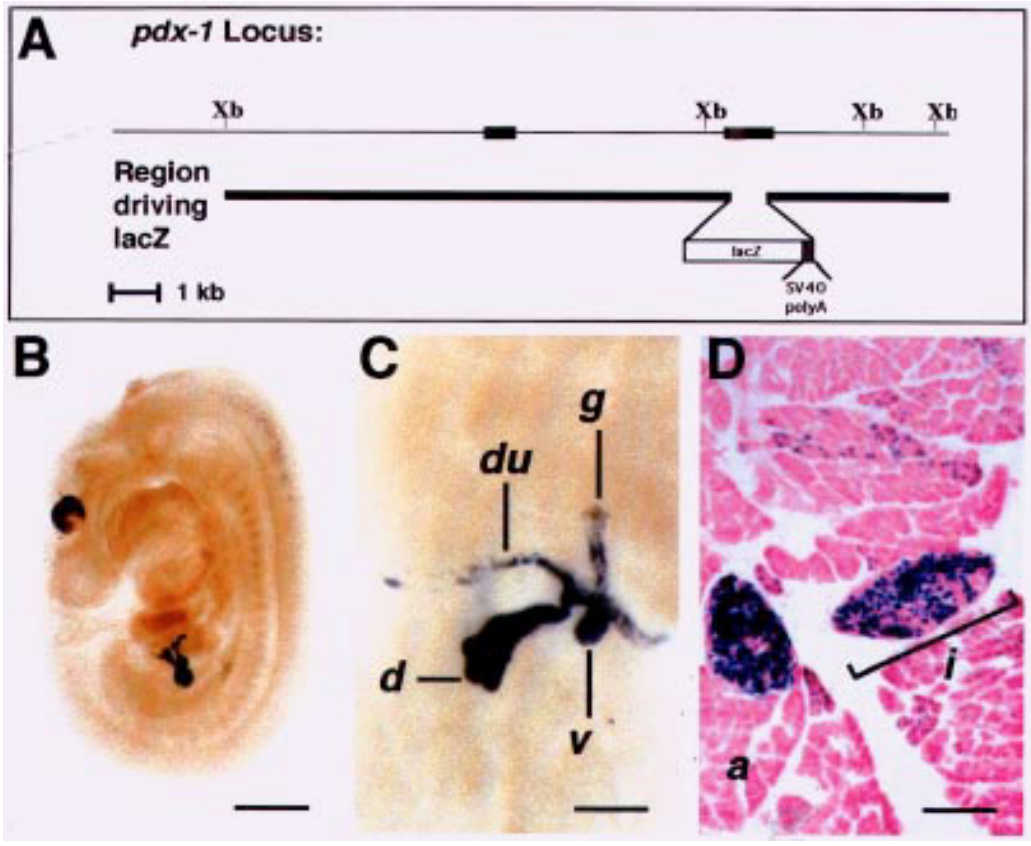

Fig. 3. Expression of a *pdx-1*^{lacZ} fusion gene in transgenic mice. *A* Diagram of the *pdx-1* locus showing location of *lac*Z insertion. Thin lines indicate noncoding sequences, and boxes represent the first and second exons. The homeodomain is located in exon 2 (hatched box). The lower thick line represents the genomic sequences in the *lac*Z-containing transgene. *B* Lateral view of an 11.5 d.p.c. transgenic embro stained in whole mount to visualize the *pdx-1*^{lacZ} expression domain (blue). Expression is detected within a restricted region of the developing posterior foregut, corresponding to the endogenous *pdx-1* expression domain. The melanized eye is visible to the upper left. Bar, 330 μm. *C* Higher-magnification view of an 11.5 d.p.c. embryo showing β-gal expression in the dorsal and ventral pancreatic buds, the rostral duodenum and common bile duct. Bar, 100 μm. *D* Section through adult pancreas counterstained with eosin. β-gal expression is seen in the majority of islet β-cells and at a lower intensity in acinar cells of some pancreatic lobes, but not others. Bar = 50 μm. Xb = *Xba*I; d = dorsal; v = ventral; du = duodenum; g = gall bladder and common bile duct; a = acinar cells; i = islet (from Wu et al., 1997).

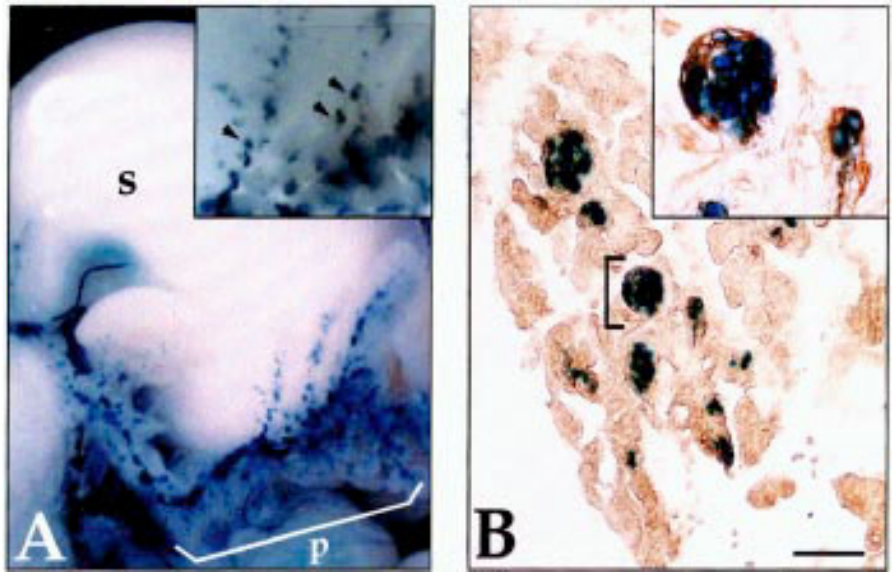

Fig. 4. Transgenic analysis of β-cell-specific *cis*-acting regulatory regions. *A* Digestive organs of a 2-day neonatal pup carrying the PB/*lacZ* reporter transgene show expression (blue) in islets within the pancreas (bracket), but not within acinar cells, stomach or duodenum. The inset shows islets at higher magnification (arrowheads). Expression is detected in islets close to the ductal epithelium, as well as those more separated from it. *B* Transgene expression colocalizes with insulin (reddish-brown) within the islets. The bracketed area is shown at higher magnification in the inset. Bar = 45 μm. s = Stomach; p = pancreas (from Wu et al., 1997).

Much of our attention has been focused on a 1 kb *PstI-BstEI* fragment (referred to as PB) that corresponds to one of three DNaseI hypersensitive sites located in the 5′ region of the gene [29]. The PB region can drive β-cell-specific expression in cell lines, and the relevance of this was confirmed by finding that PB/*lacZ* reporters are expressed islet-specifically in embryos, neonates and adults (fig. 4) [29, M.G. and C.V.E.W., unpubl. observations]. Ultimately, studying this islet-specific fragment should lead to the identification of upstream regulatory factors for *pdx-1*, functioning either during embryonic pancreas development, or involved in *pdx-1* regulation related to mature adult islet function and glucose homeostasis. Our experiments have so far identified the general endoderm transcription factor HNF3β, which is also expressed in islet cells in vivo, as a candidate transactivator of *pdx-1* in islets [29]. Within the context of the PB fragment, mutation of one of three potential HNF3β sites was associated with ∼60% reduction in reporter gene expression in β-cell lines [29]. This mutation also seems to reduce the level of β-cell-specific

*lac*Z reporter activity in transgenic mice (M.G. and C.V.E.W., unpubl. data). Overall, these results suggest that HNF3β is an important transactivator of *pdx-1* expression in β-cells, but that other factors contribute to full *pdx-1* gene activation. This leads to the hypothesis that altered HNF3β expression or reduced activity of this factor in islets would contribute to defective glucose homeostasis by affecting *pdx-1* expression. It is also possible that other MODY genes, for example HNF1α and HNF4α, together with yet to be defined gene products, will be found to interact in networks, as suggested by the work of [42]. Such regulatory networks could be highly relevant to the multigenic nature of adult onset/type 2 diabetes.

It is very likely that the core regulatory sequences involved in binding key transcriptional regulators of *pdx-1* are highly conserved between vertebrate species. Comparison of 4.5 kb of DNA sequence localized 5′ of the transcriptional start site of mouse, human and chick *pdx-1* has revealed several blocks of strong sequence identity, each ∼300 bp in length. The areas of conservation stand out dramatically from the surrounding sequences, which are much less conserved, and lie within the PB enhancer-like element described above. While some of these subregions of the PB region are conserved between all three species, others are found in mouse and human, but not chick. This finding may suggest that some mechanisms controlling *pdx-1* expression are shared between most vertebrates, while regulatory elements that are nonconserved between birds and mammals may reflect differences in pancreatic ontogeny or islet physiology. These conserved sequences are currently being tested for their ability to direct β-cell expression in mammalian cell lines and in transgenic mice. Initially, sequence motif searches have identified potential binding sites for HNF3β and other well-known endodermal transcription factors, within the conserved regions. Eventually, mutational analysis of active reporter constructs will determine sequences that bind critical regulatory factors and lead to biochemical studies of the transacting factors.

While the studies just described support the idea that HNF3β regulates *pdx-1* in β cells, its spatiotemporal expression profile excludes it from being involved in the localization of pancreatic fate to a particular region of the gut endoderm, or in the specification of the β-cell fate within the pancreas (although it was first identified as a liver-specific gene regulator, HNF3β is broadly expressed throughout the endoderm). Thus other specific factors/cofactors, or a unique region- or tissue-specific combination of more widely expressed factors, probably regulate both of these processes. Studies from several laboratories are now leading to the identification of other important transcription factors expressed in the embryonic pancreas, islet and acinar tissue, and defined mature cell types therein. Gene inactivation experiments in ES cells are beginning to define their roles. Many of these have a β-cell phenotype, although

there are variable effects on the severity and the cell types affected in the homozygous mutant animals (see article by Permutt et al., this issue). These factors are not only expressed in the pancreas. For example, *HNF3β, Pax6, Beta2/NeuroD* and *Isl1* are also expressed in neural tissue, reminiscent of the neuroendocrine and nervous system connection that initially led to the incorrect supposition that the pancreatic endocrine cells arose from migratory neural crest cells. To date, most of the individual null mutants only partially abrogate pancreas or islet development. For example, while removal of a single factor such as *PAX6* or *Beta2/NeuroD* dramatically alters islet development and/or endocrine cell proliferation, endocrine cells are found in both cases, and the acinar pancreas develops fairly normally. A striking exception is *Isl1*, which appears to be essential for differentiation of all pancreatic endocrine cells [16], as we have described above.

Just as insulin gene transcription requires the carefully regulated and combined input from a constellation of transcription factors, it now appears likely that pancreas development and β-cell differentiation are similarly dependent on the integrated function of a number of different regulators. The idea that pancreatic cell type specification may not operate through a simple set of master switches involving individual factors has relevance to ideas of how we might dominantly convert pluripotential stem cell precursors toward pancreatic fates for diabetes therapy via transplantation. In the meantime, another implication is that the number and type of gene products identified as contributing to MODY and adult onset diabetes will continue to increase. Moreover, we may find that the severity of the disease is correlated with different coinherited combinations of genetic lesions corresponding to multiple different endoderm and islet regulatory factors.

Acknowledgments

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C.V.E. Wright, DPhil, Department of Cell Biology, Vanderbilt University Medical Center, Nashville, TN 37232 (USA)
Tel. +1 615 343 8256, Fax +1 615 343 4539, E-Mail wrightc@ctrvax.vanderbilt.edu

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Molecular Mechanisms of PDX-1 Regulation

Ulupi S. Jhala, Seema Sharma, Sanjoy Dutta, Marc Montminy

Joslin Diabetes Center, Research Division, Department of Cell Biology,
Harvard Medical School, Boston, Mass., USA

A growing body of evidence in recent literature indicates that the homeobox transcription factor PDX-1 plays a key role in pancreatic endocrine function. Typically, most members of the homeobox class of transcription factors serve two distinct functions. First, during embryonic development, the spatial and temporal expression of these proteins is critical for patterning and cell fate determination. Second, the homeobox proteins regulate cell specific gene expression postnatally thus becoming key regulators of specialized cellular function.

Similar to other proteins in its class, PDX-1 has two distinct functional roles. PDX-1 is a critical regulator of pancreas development. Its early expression at e8.5 in mouse embryonic endoderm is thought to initiate a cascade of sequentially expressed genes that lead to formation of the pancreas [1]. However, as the pancreas develops, PDX-1 gene expression becomes progressively restricted to the insulin secreting β cells where it is thought to regulate glucose-induced gene expression. Indeed, a failure to express PDX-1 protein results in pancreatic agenesis both in humans and in mice [2, 3]. Furthermore, haploinsufficiency of PDX-1 results in early onset diabetes which has lead to the identification of *PDX-1* as the MODY4 gene [4–6].

In addition to PDX-1, other homeobox proteins including Isl-1, Pax4, Pax6, nkx6.1, and nkx2.2 are expressed in the pancreas [7–11]. Despite the promiscuous binding of homeobox proteins to TAAT box motifs in vitro, PDX-1 and the other homeobox transcription factors exhibit remarkable binding specificity and highly selective gene activation in vivo [12]. Taken together, these observations are strongly indicative of a higher order of regulation of PDX-1, to achieve its highly specific effects on pancreas development and

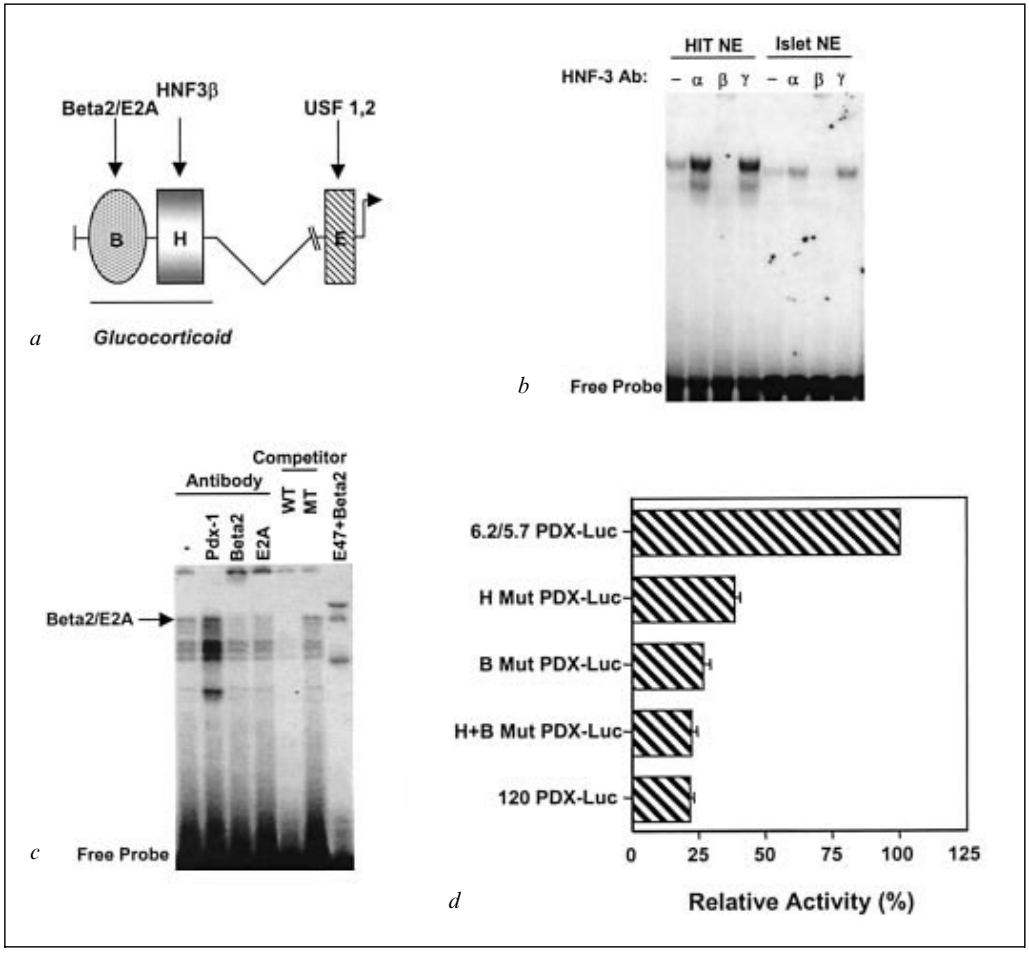

Fig. 1. a Schematic of the functional elements of the rat PDX-1 promoter: Three functional elements are depicted. The proximal E-box denoted by the hatched rectangle is located at –104 bp from the transcription initiation site and binds the bHLH protein USF. The distal islet specific enhancer (–6.2–5.7) consists of two important sites, the HNF3β-binding site denoted by the shaded H box and the Beta2/E2A-binding site denoted by the stippled oval labelled B. The distal enhancer is also the region through which dexamethasone mediates its inhibitory effects on the *PDX-1* gene. *b* Specific binding of forkhead protein HNF3β to H box of distal enhancer of rat PDX-1 gene: Gel mobility shift analysis of ^{32}P labeled H box incubated with nuclear extracts from HIT insulinoma cells or isolated rat islets. Antibodies to HNF3 α, β or γ were included. Supershift of DNA protein complex is solely in the presence of HNF3β antibody indicates specific binding by HNF3β versus other members of the family. *c* Distal E-box binds islet-specific bHLH transcription factor Beta2: The distal B element (E-box) was ^{32}P radiolabeled and incubated with nuclear extracts from HIT insulinoma cells, in the presence of antibodies to PDX-1, Beta2 or E2A. Note the

insulin gene activation. We consider the following three mechanisms: (1) Tissue specific regulation of *PDX-1* gene expression. (2) Modulation of DNA binding specificity of PDX-1 protein. (3) Signal dependent activation of PDX-1-mediated target gene expression.

Tissue Specific Regulation of the *PDX-1* Gene

Developmentally, PDX-1 is expressed in the dorsal endoderm at day e8.5. As embryogenesis proceeds, PDX-1 expression becomes progressively restricted to the endocrine pancreas, specifically to the insulin-secreting β cells and somatostatin-secreting δ cells suggesting a dynamic regulation of *PDX-1* gene expression during development [1]. Duodenal expression of PDX-1 has been detected; however its role in duodenal function and development remains to be determined. The complex expression profile of PDX-1 in throughout pancreas during development, indicates a mechanism that activates initial expression of *PDX-1* gene in both exocrine and endocrine progenitor cells, followed by either an active progressive repression of *PDX-1* gene in exocrine cells or an attrition of transient cells via apoptosis.

Although the complex dynamic profile of *PDX-1* gene expression has been fairly well characterized, the specific hormonal and developmental cues that regulate *PDX-1* gene expression are not yet understood. We have previously reported that 6.5 kb of the rat *PDX-1* sequence upstream from the transcription initiation site is sufficient to direct expression of a β-galactosidase reporter to the islet and intestinal duodenal cells [13]. Two regions are of particular importance for appropriate expression of the *PDX-1* gene. As depicted in a schematic of the PDX-1 promoter (fig. 1a), the proximal promoter has an E-box element (–104), that binds the ubiquitous helix-loop helix protein, USF. This site is necessary for expression of the *PDX-1* gene. In fact, infection of islet cell lines with adenovirus expressing dominant negative USF results in a dramatic reduction in PDX-1 mRNA (Dutta and Montminy, unpublished observations). Although recent studies have implicated USF-2 as a mediator

supershift of Beta2/E2A complex only in the presence of Beta2 or E2A antibodies. Specificity of binding was also assessed by disappearance of the complex in the presence of wild-type (WT) cold excess competitor DNA but not Mutant (MT) DNA. The last lane shows binding of recombinant Beta2 and E2A to the B element. *d* Both B and H elements are required for full PDX-1 enhancer activity: Luciferase activity derived from wild-type or mutant distal enhancer constructs with mutations in the B, H or both elements, upstream of a minimal PDX-1 promoter, transfected into HIT insulinoma cells are depicted in a bar graph. Note the marked attenuation of reporter activity with a mutation in either the B or H element.

of glucose responsive gene expression in the liver [14], there is no evidence to suggest that USF-2 can activate *PDX-1* gene in a glucose-dependent manner. While the precise role the proximal E-box in *PDX-1* gene expression is not clear, other nuclear factors, most notably myc and Max are capable of binding this site with high affinity in vitro. As the myc gene product is typically present in proliferating cells, and *PDX-1* gene appears to be abundantly expressed in pancreatic ductal cells, it is tempting to speculate that during rapid proliferation of pancreatic progenitor cells, E-box activity may play a regulatory role in *PDX-1* gene expression.

In addition to the proximal E-box, we have characterized a distal (–6.2 to –5.7) composite enhancer, which directs *PDX-1* gene expression to islet cells via two functional elements – an HNF3β-binding site, and an E-box that binds the islet specific helix-loop helix protein BETA2 (fig. 1b and 1c). Like the proximal E-box element, both upstream binding sites are necessary for full promoter activity; and a mutation in either site is sufficient to abolish PDX-1 promoter activity (fig. 1d).

In a related report, Wu et al. demonstrated that a region of the mouse *PDX-1* (–2560 to –1880) gene that is sufficient to direct robust β-galactosidase reporter activity to the islets of transgenic mice, shows a substantial attenuation of reporter expression when mutated for an HNF3β-binding site [15]. Interestingly, HNF3β is first expressed in node and notochord as well as the progenitor cells of definitive endoderm and is considered to be instrumental in the initiation and maintainence of endodermal lineage [16]. In fact HNF3β null embryos die early in embryogenesis, just prior to endodermal development [17, 18]. As development proceeds the expression of HNF3β persists in other organs of endodermal origin such as lung and liver [19]. HNF3β pattern of expression during pancreas development has not been well documented, however studies on adult rats indicate that in pancreas, HNF3β expression is largely nuclear and is restricted to the insulin-secreting β cells (Jhala and Montminy, unpublished observations). Furthermore, coincident expression patterns of HNF3-β protein and *PDX-1* gene, are supportive of a regulatory role for HNF3β in *PDX-1* gene expression.

While the HNF3 site appears to direct expression of *PDX-1* gene to the endoderm, regulation by Beta2, an islet-specific specific member of the helix loop helix family of trancriptional regulators may further restrict expression of *PDX-1* gene to insulin-secreting β cells. Within the distal enhancer, in close proximity to the HNF3β site is an E-box that binds a Beta2/E2A heterodimer. Beta2 has been demonstrated to regulate insulin gene expression in vitro [20] as well as in vivo. Targeted disruption of both alleles of the Beta2 gene results in abnormal islet morphology, with a failure of islets to cluster, reduced β cell mass, severe diabetes and perinatal death [21]. In an apparent contradiction

these animals did appear to express *PDX-1* as well as insulin genes, however a profound decrease in β cell mass may underscore the importance of Beta2 in expression of PDX for pancreatic development. Furthermore, Beta2 as well as PDX-1 binding sites are required for full insulin promoter activity. Recent studies of diabetic familial pedigrees, have linked mutations in the coding sequence of Beta2 to the incidence of familial MODY and type 2 diabetes (Malecki, Jhala, Montminy and Krolewski, unpublished observations).

While the HNF3β and Beta2 sites in the composite enhancer are essential for *PDX-1* gene expression, the specific mechanisms by which these and other uncharacterized regions of the promoter integrate extracellular signals to regulate gene expression is largely unknown. HNF3 belongs to the winged/forkhead family of DNA-binding proteins. Forkhead (FKH) family proteins play central roles in cellular differentiation, embryonic development and organogenesis [22]. Recent studies have also identified some FKH proteins as downstream mediators of TGFβ and insulin signaling pathways [23–26]. Further analyses of HNF3β and Beta2 protein activities may reveal other cues that modulate the expression of *PDX-1* gene.

In addition to the insulin and TGFβ pathways, the activity of the HNF3 family of proteins also intersects with glucocorticoid signaling pathway. Specifically, in the context of various genes, HNF3α and HNF3β have been shown to either facilitate or inhibit glucocorticoid action [27–30]. Coincident with their inhibitory effects on islet maturation, glucocorticoids (dexamethasone) treatment results in a 10–20 fold inhibition of the rat mouse *PDX-1* genes. In case of the rat *PDX-1* gene these effects map to the promoter regions that contain the HNF3 binding sites essential for islet-specific (fig. 2a and 2b). Interestingly, the rat PDX-1 promoter does not contain a GRE; however the inhibitory effects of glucocorticoids can be mapped to the HNF3 site. Overexpression of HNF3β can reverse a majority of the dexamethasone-mediated inhibition of the *PDX-1* gene [30]. The absence of a GR binding site argues against competition between GR and HNF3β for promoter occupancy. Rather, it is possible that GR and HNF3β may putatively compete for a common coactivator protein, such as p300/CBP.

Studies reveal that PDX-1 is a potent transcriptional activator of β cell specific genes such as glucokinase, Glut-2 and insulin [31–33]. It is possible that the repressive effects of glucocorticoids in *PDX-1* gene activation also downregulate PDX-1 target genes. Studies show that glucocorticoids inhibit glucose-stimulated insulin secretion from isolated islets [34]. Experiments from our laboratory indicate that dexamethasone affects islet gene expression, at least in part through its effects on overall PDX-1 expression. Prolonged treatment (36–48 hrs) of islet cell lines with dexamethasone showed a virtual shutdown in the expression of PDX-1 mRNA, a 70–80% drop in PDX-1 protein

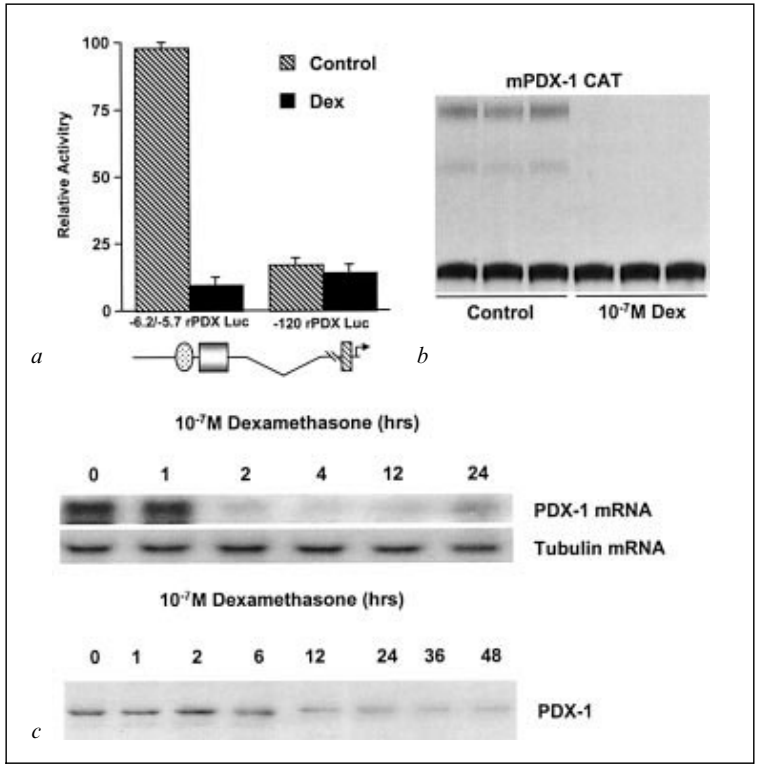

Fig. 2. a Inhibition of distal enhancer (6.2–5.7) of rat PDX-1 promoter by dexamethasone treatment: Wild-type constructs of the islet-specific distal enhancer including the B and H element (depicted in the schematic of rat PDX-1gene) or the minimal promoter (120 Luc) of the PDX-1 gene were transfected into HIT cells. Compared to the minimal promoter, treatment with 10^{-7} M dexamethasone shown using dark bars, resulted in 10-fold repression of the PDX-1 enhancer. Ethanol treatment shown as the hatched bars serve as a control. *b* Dexamethasone-mediated inhibition of the islet-specific enhancer of mouse PDX-1 gene: CAT reporter activity derived from HIT cells transfected with the islet-specific enhancer of the mouse PDX-1 gene (–2560–1880) in the presence or absence of 10^{-7} dexamethasone is shown (in triplicates). Similar to the rat promoter, dexamethasone treatment resulted in profound inhibition of the mouse PDX-1 gene expression. *c* Time course of dexamethasone (10^{-7} M) treatment on PDX-1 mRNA and PDX-1 protein in HIT cells: Northern blot analysis (upper panel) of 20 μg of total RNA from HIT cells treated with dexamethasone resulted in a marked suppression of PDX-1 mRNA within 2 h of treatment and remained suppressed even at 24 h. Tubulin which served as a control, remains unchanged. Western blot analysis (lower panel) was performed on 20 μg of whole cell lysates from HIT cells treated with dexamethasone. PDX-1 levels were detected using anti-PDX-1 antisera, and started to diminish at 24 hrs and continue to fall even at 48 hrs of dexamethasone treatment.

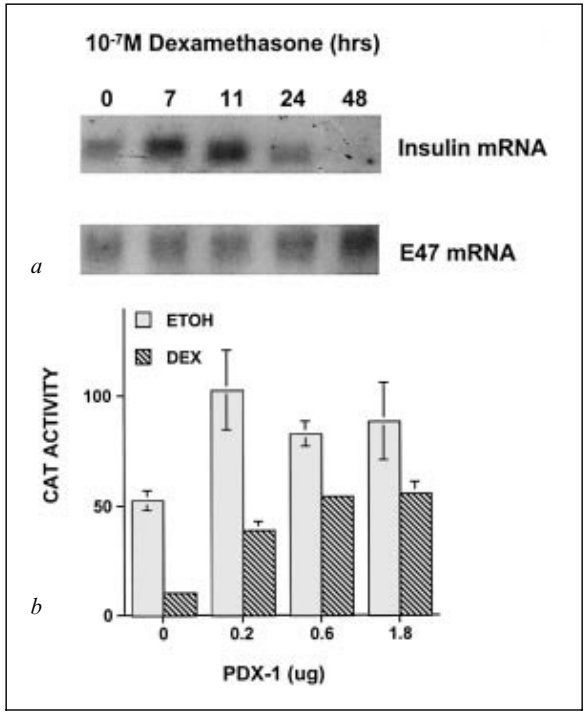

Fig. 3. a Time course of dexamethasone (10^{-7} M) treatment on PDX-1 mRNA and PDX-1 protein in HIT cells: Northern blot analysis of 20 μg of total RNA from HIT cells treated with dexamethasone resulted in a marked suppression of insulin mRNA around 24 h of treatment and remained suppressed even at 48 h. E47 (control) mRNA levels remains unchanged. *b* PDX-1 mediates reversal of dexamethasone-inhibited insulin gene activity: Bar graph of reporter activity derived from HIT cells transfected with Ins1-CAT and increasing amounts of PDX-1, in the presence of ethanol (stippled bars) or 10^{-7} dexamethasone (hatched bars). Note the restoration of dexamethasone-inhibited insulin gene expression with PDX-1.

levels (fig. 2c), and a concomitant drop in insulin mRNA levels (fig. 3a). Similarly, in transfection experiments carried out in islet cell lines, insulin reporter gene activity also was inhibited five-fold by dexamethasone. A majority of this inhibition was rescued by the overexpression of PDX-1 protein (fig. 3b), indicating that some of the effects of dexamethasone on the islet phenotype are exerted at the level of transcriptional activities of PDX-1 protein.

Based on our results, glucocorticoids inhibit PDX-1 gene and thereby inhibit its ability to activate target genes. The overall effect manifests as a suppression of the islet phenotype. Although the mechanisms governing islet differentiation are not understood, in AR42J cells, an amphicrine pancreatic

cell line, glucocorticoids have been shown to promote acinar cell differentiation [35–36]. Furthermore, a post-natal surge in circulating glucocorticoids is correlated with an expansion of the exocrine pancreatic compartment [37–38]. Taken together, these data indicate that the islet-specific enhancer may be pivotal in the integration of both positive and negative signals that regulate *PDX-1* gene expression and subsequent outcomes.

Modulation of DNA Binding Specificity of PDX-1 Protein

The critical role of PDX-1 in pancreatic morphogenesis is underscored by a virtual failure of the pancreas to develop beyond a rudimentary bud in PDX-1 null mice. However, the known targets for PDX-1 action include insulin, Glut-2 and glucokinase and somatostatin genes, all of which are primarily expressed in the mature islet, and have no known function in the morphogenesis of pancreas. Early targets for PDX-1 gene in pancreatic organogenesis are largely unknown.

During development, rapid changes in gene expression, result in dramatic phenotypic changes, most of which have major long-term implications on the developing embryo. Hence specificity of target gene activation is critical. The critical importance and need for target specificity, coupled with frequent occurrence of TAAT motifs in the genome (1 in every 256 bases), suggests that DNA binding specificity of homeobox proteins may be modulated by interaction with other proteins. Indeed, PDX-1 and other homeobox proteins of the antennaepedia class interact with a class of atypical homeodomain proteins known collectively as the TALE (Three Amino acid Loop Extension) proteins. PDX-1 and PBX (Pre- B cell Hox) proteins are prototypes of these two classes of proteins, respectively [40].

One of the goals of our laboratory has been to understand the molecular mechanisms underlying the regulation of the somatostatin (SMS) gene, which is expressed in the β cells of the islets of Langerhans. The SMS gene is tightly regulated by at least three response elements, including a cAMP response element and two other tissue specific elements called TSE-I and TSE-II [41]. As shown in figure 4a the TAAT motif constituting TSE-I serves as a monomeric PDX-1 binding site, much like the P box and Flat elements of the insulin gene. However TSE-II contains two tandem TAAT motifs, which serve as a high affinity binding site for PDX-1/PBX heterodimers. This interaction is highly specific and is mediated by a conserved pentapeptide sequence FPWMK within PDX-1, a motif that is conserved in a large subset of HOX proteins. Additional residues in the N-terminal arm of the homeodomain of PDX-1 also are involved in this interaction. Mutation of this pentapeptide motif is

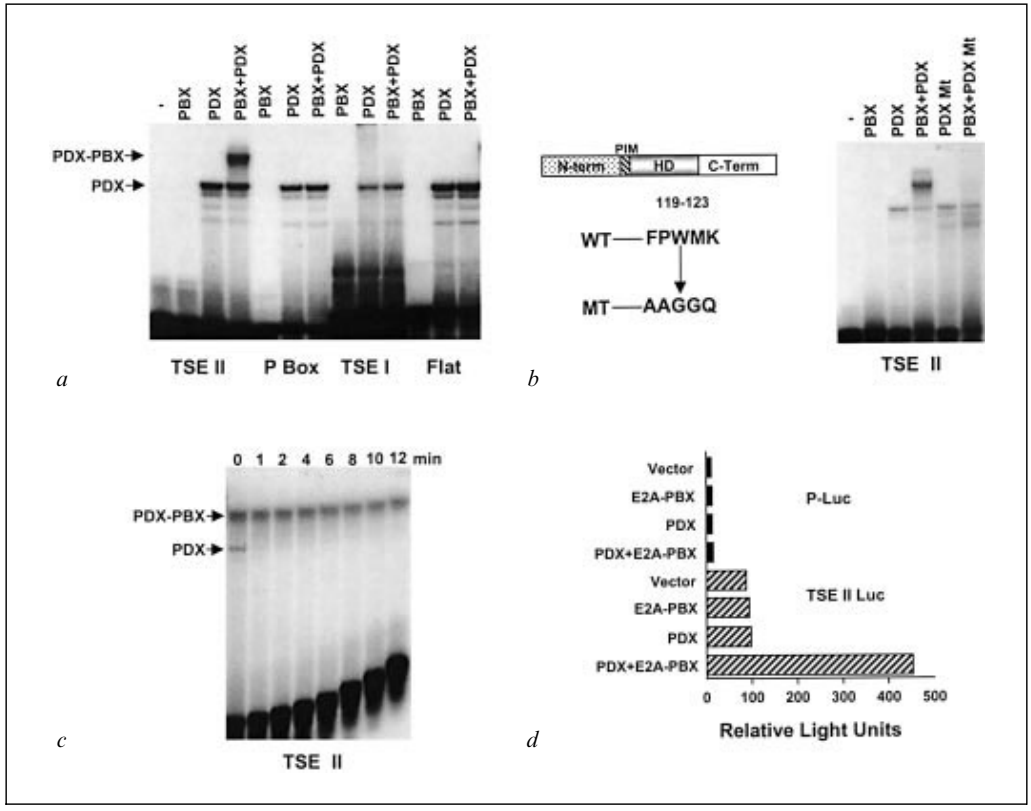

Fig. 4. a Analysis of PDX-1/PBX heterodimer formation on somatostatin and insulin promoter sequences. Gel retardation assays using labeled oligonucleotide probes including TSE II-a somatostatin promoter sequence that binds PDX-1/PBX heterodimer, P box and Flat elements from insulin gene and TSE I element from somatostatin gene, that bind PDX-1 monomer, were performed using recombinant PBX and PDX-1. Monomeric and dimeric complexes of PDX-1 and PBX/PDX-1 are indicated. Note formation of the dimer only on the TSE II element. *b* A conserved pentapeptide motif in PDX-1 is critical for cooperative binding with PBX. A schematic of the PDX-1 protein is indicated with the conserved pentapeptide denoted as PIM (PBX-Interaction Motif). Mutation of the conserved FPWMK motif to AAGGQ destroys the cooperativity of PDX-1 interaction with PBX as seen by the disappearance of the upper band (compare lane 3 to lane 5). PDX-1 binding (lower band) remains intact. *c* PBX-1 stabilizes PDX-1 binding to TSE II. Shown are results of off-rate analysis PDX-1 and PDX-1/PBX complexes with TSE II oligonucleotide as probe. Time (in minutes) after addition of excess unlabelled oligonucleotide is indicated. Note the rapid disappearance of PDX-1 complex compared to PDX-1/PBX complex. *d* PDX-1 and PBX synergistically activate a subset of promoter sites. Shown are results of a transient transfection assay with PDX-1 and PBX-E2A effector plasmids as indicated, using either the insulin gene PDX-1 binding motif or TSE II to drive a reporter gene. Note the ability of synergistic activation of TSE II-Luc versus P-Luc.

sufficient to abolish PDX-1/PBX interaction, and dimer formation on TSE-II. Furthermore, as shown in figure 4c, not only does PDX-PBX heterodimerization increase the complexity in DNA binding specificity; it also markedly increases the DNA binding affinity of PDX-1 by decreasing the off-rate of the heterodimer from TSE-II by fifteen-fold as compared to the off-rate of PDX-1 monomer binding to TSE-II site. In transfection assays, PDX-1 and PBX can synergistically activate a TSE-II driven reporter, but have little effect on a monomeric PDX-1-binding site, indicating that PDX-PBX heterodimers not only increase binding specificity and affinity but can also cooperatively activate transcription.

The complexity of regulation by dimerization and target selection by PDX-PBX proteins is multiplied by the findings that PBX proteins themselves interact with other subgroup of the family of TALE proteins which include Prep-1 (PBX Regulating Protein 1), Meis 1–3 and Hth (homothorax) [40, 41]. The Prep-1/Meis proteins interact directly with PBX proteins in the absence of DNA, and modulate nuclear translocation of PBX proteins in response to extracellular signals. The ability of Prep-1 proteins to bind and sequester PBX likely alters the relative concentration of protein available for interaction with PDX-1 [43]. Furthermore, PBX/Prep1 dimers also bind to distinctly different DNA target sequences as in case of the somatostatin promoter [44]. These changes in DNA binding site selection and affinity would result in the activation of a completely different set of targets.

While most of these targets are yet to be discovered, studies suggest that regulation of β-cell specific genes may result from the the action of PDX-1 monomers rather than through dimeric and trimeric interactions with PBX and Prep1 proteins [44–45]. By contrast, somatostatin regulation in δ cell lines and elastase gene regulation in acinar cell lines appears to be mediated by trimeric complexes including PDX-1, PBX and Prep-1 proteins. Thus, the dynamic interaction of PDX-1 in transcriptional complexes with varying partners or cofactors that arise in response to different extracellular signals may provide the basis for divergence of islet and acinar cell lineages as well as maintainence of the post-differentiation phenotype of the pancreatic cells types.

Signal-Dependent Activation of PDX-1-Mediated Target Gene Expression

The predominant expression of PDX-1 in mature β cells, and its ability to regulate insulin gene expression implies a regulatory role for PDX-1 in glucose homeostasis. In fact the association of PDX-1 mutations with its early

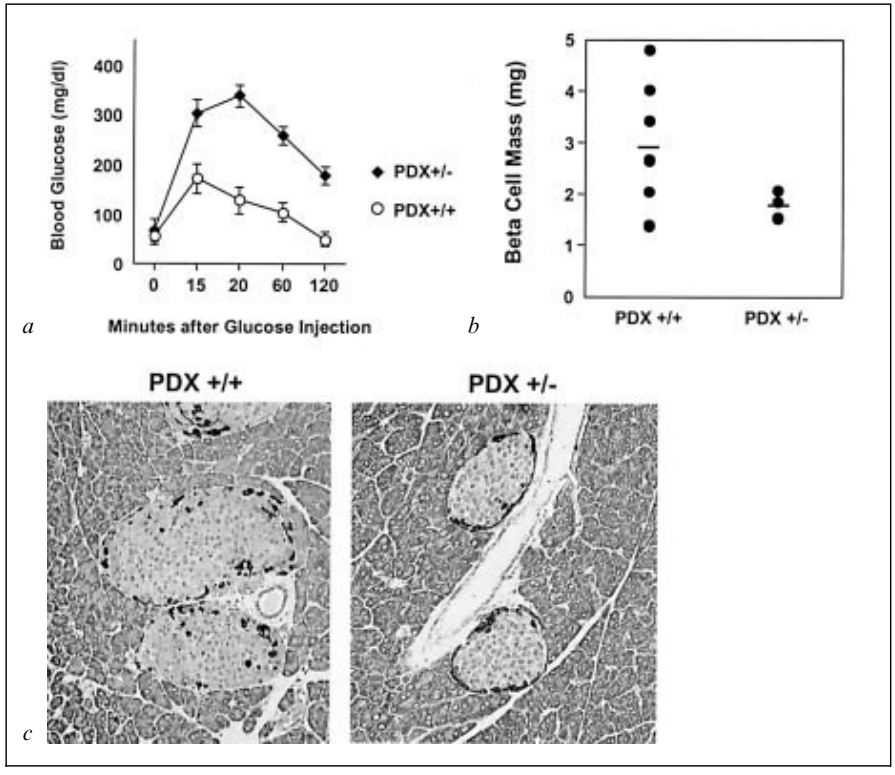

Fig. 5. a Glucose tolerance is impaired in PDX +/– compared to PDX +/+ mice. Glucose tolerance tests were conducted on fasted black Swiss mice that are heterozygous (denoted by closed diamond) or homozygous for PDX-1 (denoted by open circles). Mean blood glucose values over time are shown. *b* Scatter diagram of total islet mass from PDX-1 +/+ versus PDX-1 +/– animals. PDX-1 +/– animals have lower mean islet mass per pancreas; however the difference was not statistically different from that of the wild-type mice. *c* Representative islets from PDX +/+ and PDX +/– mice. Pancreas from 15-week-old mice were immunostained with combined antibodies raised against glucagon, somatostatin, and pancreatic polypeptide to show the mantle of islet non-β cells around the core of β cells. Magnification = 50 μm.

onset diabetes in several families has led to classification of *PDX-1* as the MODY4 gene.

Studies on PDX-1 +/– heterozygous mice in our laboratory revealed a marked impairment in glucose tolerance following an intraperitonial glucose bolus with significantly high blood glucose levels at 30 minutes post-load that remained elevated throughout the duration of the glucose tolerance test (see fig. 5a). Although individual islets appeared to be smaller in size, the average

β-cell mass in PDX+/– mice was not decreased to a significant extent compared with the PDX+/+ mice (fig. 5b and 5c) [4]. Our results were corroborated by those of another study in mice, where the inactivation of PDX-1 gene in 80% of the β cells using the Cre-Lox system resulted in overt diabetes with severe elevation of fasting and non-fasted glucose levels, markedly reduced insulin secretion and elevated glycosuria. This manifestation of diabetes was traced to the nearly complete loss of expression of Glut2-glucose transporter accompanied by a loss of insulin expression in the β cells. The mice demonstrated some decrease of β cell mass (up to 40%), but the loss of insulin production (90%) was disproportionately high for the loss of β cell mass suggesting that PDX-1 is important both for the growth, as well as the functional integrity, of insulin-secreting β cells [6].

While our studies clearly indicate a pivotal role for PDX-1 in maintaining glucose homeostasis in the whole animal, the molecular and biochemical underpinnings for the metabolic manifestation of these genetic defects remain to be determined. It has been suggested that PDX-1-mediated transcriptional regulation of Glut-2, glucokinase and insulin genes, may be central to the diabetic phenotype in PDX-1 heterozygous animals.

Several studies have implicated PDX-1 as a direct mediator of glucose induced gene transcription in insulin-secreting β cells. PDX-1 has been proposed to regulate transcription of Glut2, glucokinase and insulin genes, the products of which constitute a critical axis in Glucose Stimulated Insulin Secretion and Synthesis (GSISS) [46]. Despite the lack of clarity with respect to the mechanism by which glucose activates kinase pathways, PDX-1 appears to be phosphorylated in response to glucose in both islets as well as β cell lines. Phosphorylation of transcription factors represents a pervasive mechanism for gene regulation in reponse to extracellular signals. Phosphorylation may modify activity of transcription factors by one or more the following events: (1) nuclear/cytoplasmic targeting; (2) change in DNA binding; (3) transactivation.

Two groups of researchers have demonstrated independently, glucose-induced nuclear translocation and DNA binding of PDX-1 protein to target sequences [47–48]. Our observations (Jhala and Montminy, unpublished observations) as well as those by Peterson et al. suggest that the amino terminal of PDX-1 (1–149) protein, when fused to Gal4 DNA-binding domain, is sufficient to confer glucose inducible transcription to a Gal4-driven reporter gene [49]. Thus while evidence for phosphorylation and glucose-induced transcriptional activity of PDX-1 protein has been demonstrated, knowledge of the activating signalling pathways as well as the precise molecular events that trigger the cascade of kinases, remain elusive [49–50]. Several recent studies in animal models have demonstrated the importance of insulin receptor tyrosine

kinase signaling cascades in the development and function of β cells. Both β-cell specific insulin receptor knockout animals and animals heterozygous for insulin receptor substrate-2 (IRS-2) display impaired glucose tolerance of varying severity [51–52]. These data indicate that insulin/IGF-1-dependent tyrosine kinase cascades are necessary for glucose-dependent gene activation in the β cell, perhaps by autocrine effects of secreted insulin. It is possible that PDX-1 is a substrate for these kinase cascade(s).

In vitro studies using isolated islets or β cell lines have implicated different kinase pathways including the PI3-kinase, MAP/ERK2 kinase and the p38-SAP/JNK kinase in the regulation of glucose-dependent phosphorylation and glucose inducible, PDX-1-dependent gene activation [53–55]. The discovery of the specific kinase(s) that mediate glucose-induced phosphorylation, and the subsequent specific events that allow target gene activation via PDX-1 and other transcription factors, will provide a broader understanding for drug design in the treatment of diabetes mellitus.

Acknowledgments

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Marc Montminy MD, PhD, Joslin Diabetes Center, Boston MA 02215 (USA)
Tel. +1 617 735 1926, Fax +1 617 735 1928

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Role of Homeodomain Transcription Factor IPF-1 in the Pathogenesis of Diabetes mellitus

Joel F. Habener[a], *Doris A. Stoffers*[a, c], *Dariush Elahi*[a], *El Habib Hani*[b], *Philippe Froguel*[b]

[a] Laboratory of Molecular Endocrinology, Massachusetts General Hospital, Howard Hughes Medical Institute, Harvard Medical School, Boston, Mass.,
[b] CNRS-UPRES A8090, Institute Pasteur of Lille, Lille, France;
[c] Division of Endocrinology, Diabetes and Metabolism, University of Pennsylvania School of Medicine, Philadelphia, Pa., USA

Diabetes mellitus is a severe disabling disease that is rapidly increasing in prevalence throughout the populations of the world. It is estimated that 100 million or more individuals are afflicted with diabetes and are destined to a premature death due to accelerated cardiovascular disease or renal failure [4, 5]. Diabetes results from a deficiency of insulin production by the β-cells of the endocrine pancreas in the amounts required to meet the body's needs. Insulin deficiency is either absolute as in type 1 juvenile diabetes or relative as in type 2 maturity onset diabetes [4].

The underlying causations of diabetes are poorly understood but clearly involve a complex interplay of genetic background and environmental influences. Approximately 90% of all diabetes is type 2 and is characterized as a polygenic disorder with complex inheritance. This circumstance has prompted a search for specific diabetes genes responsible for the causation of diabetes. During the past five years at least five such diabetes genes have been identified as causes of type 2 diabetes, thereby defining a subset of diabetes known as monogenic, or in some cases digenic, diabetes as the disease is inherited in an autosomal dominant fashion with variable penetrance and allelic linkage (compound heterozygosity) (reviewed in [6]).

Historically, one of the first familial forms of monogenic diabetes was discovered by S. Fajans in a large extended family and was so designated

Table 1. Gene mutations resulting in maturity onset diabetes of the young (MODY)

Transcription factors	Mutant gene
MODY1	HNF-4α
MODY3	HNF-1α
MODY4	IPF-1
MODY5	HNF-1β
Enzyme	
MODY2	Glucokinase

So far five genetic loci have been identified that are linked to familial early onset type 2 diabetes. Four of the five genes encode transcription factors important for proper islet cell functioning (HNF-1α, HNF-4α, HNF-1β, and IPF-1). The remaining gene encodes glucokinase, a rate-limiting enzyme involved in glucose sensing and metabolism in pancreatic β-cells.

maturity onset of diabetes of the young (MODY) because of the relatively early age of onset of the type 2 diabetes [7]. Subsequently four additional genetic loci were discovered and determined to be responsible for MODY (table 1). By using positional cloning approaches and candidate gene approaches the proteins encoded by the genes were determined. Notably, four of the five MODYs are due to mutations in transcription factors: HNF-1α [8], HNF-1β [9], HNF-4α [8], and IPF-1 (also known as IDX-1, PDX-1, STF-1) [2]. Members of the MODY1 family originally described by Fajans et al. [7] carry mutations in HNF-4α. The fifth MODY locus (MODY2) encodes glucokinase, a rate-limiting enzyme for glucose metabolism in β-cells [10]. The association of familial late onset diabetes with the inheritance of a mutation in the transcription factor IB-1 is described by Waeber et al. in this volume.

Homeodomain Transcription Factor IPF-1 Required for Pancreas Development

The homeodomain transcription factor IPF-1, also known as IDX-1/PDX-1/STF-1, was cloned from pancreatic islet cell lines by three groups

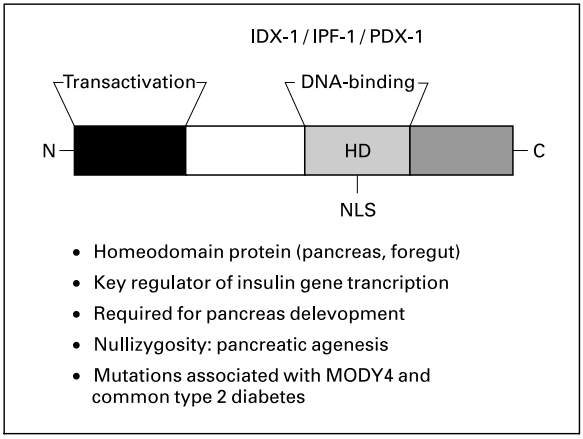

Fig. 1. Homeodomain transcription factor IDX-1/IPF-1/PDX-1. The protein of 283 amino acids consists of a homeodomain (HD) that is highly conserved in the large family of homeodomain proteins important for development. The homeodomain contains the DNA-binding region and a nuclear translocation signal (NLS) required for transport to the nucleus. The transcriptional transactivation domain resides at the amino terminus (N). A cryptic transactivation domain is also located in the C-terminal region. IPF-1 is required for pancreas development as disruption of the gene in mice and humans results in pancreatic agenesis. Hemizygosity at the *ipf*-1 locus results in diabetes in mice and in members of a family with autosomal dominant inherited diabetes (MODY4). Several missense mutations in IPF-1 are associated with glucose intolerance or late onset type 2 diabetes.

independently [11–13] (fig. 1) (for reviews see [3, 14–17]). *ipf*-1 and *pdx*-1 are the official nomenclature for the human and mouse gene loci, respectively. IPF-1 is one of an estimated 60–70 proteins that serve as transcription factors and are related by the highly conserved homeodomain required for DNA-binding and protein-protein interactions [18]. Homeodomain proteins, along with bHLH (basic helix-loop-helix) proteins, play critical roles in the spatial and temporal patterns of gene expression during development and are responsible for determination of the body plan and organogenesis [19]. After development is completed homeodomain proteins typically function as important transcriptional regulators of key cell-specific genes. IPF-1 belongs to a Para-Hox group of three proteins including also Cdx-2 and Gsh-1 encoded by closely spaced loci on chromosome 13q12.1 (human) [20]. Cdx-2, IPF-1, and Gsh-1 are expressed in the hindgut, foregut and forebrain, respectively.

The expression of *ipf*-1 appears to be restricted to the developing endodermal epithelium of embryonic foregut destined to become the pancreas [21]. When pancreas development is completed shortly before birth, IPF-1

expression occurs predominantly in the β-cells and δ-cells (not the α-cells) of the endocrine pancreas, the mucosal epithelium of the distal stomach and proximal duodenum, and occasional epithelial cells located in the pancreatic ducts and the exocrine pancreas.

Homozygosity for an Inactivating Mutation of the *ipf*-1 Gene Results in Pancreatic Agenesis in Mice and Humans

The importance of IPF-1 in pancreas development is underscored by the findings that a targeted disruption of the *ipf*-1 gene in mice results in a phenotype of pancreatic agenesis [22]. The striking phenotype of pancreatic agenesis in *ipf*-1 null mice prompted a candidate gene approach to examine a child born without a pancreas for the existence of inactivating *ipf*-1 mutations [3]. Analyses of the nucleotide sequence of the *ipf*-1 gene in the child with pancreatic agenesis revealed homozygosity for a deletion of a cytosine in a stretch of six cytosines at codon 63. The deletion of the cytosine results in a frameshift for translation from open reading frame-1 (ORF-1) to ORF-2 and a premature termination of translation at codon 122 (fig. 2). This circumstance predicted the formation of a biologically inactive form of IPF-1 devoid of the DNA-binding homeodomain and unable to enter the nucleus, because the nuclear localization signal resides within the homeodomain. Remarkably, in addition to the formation of an inactive truncated N-terminal form of IPF-1 as a result of the Pro63fsdelC mutation, a C-terminal isoform of IPF-1 is produced lacking the N-terminal transactivation domain. This somewhat unusual circumstance occurs by way of the activation of an internal cryptic translation initiation codon (AUG codon 22) that reinitiates translation of IPF-1/Pro63fsdelC mRNA in ORF-3 and then switches back to ORF-1 of the wild-type IPF-1 when encountering the cytosine deletion at codon 63. Such a reinitiation of translation in response to premature translational termination is well recognized in the literature and is a consequence of the premature release of ribosomes from the mRNA, thereby allowing for the reassembly of a translation initiation complex at the cryptic internal initiator AUG codon [23, 24]. The consequence of the internal translation is the production of a potential dominant negative isoform of IPF-1 since the internally translated protein retains the homeodomain required for binding to DNA and nuclear localization, but lacks a transactivation domain (fig. 3).

The child with pancreatic agenesis is homozygous for the Pro63fsdelC mutation in IPF-1, and therefore both parents are heterozygous carriers of the mutation. Notably, the father and the mother of the child were diagnosed as having diabetes at ages 17 and 24 years, respectively. A further examination

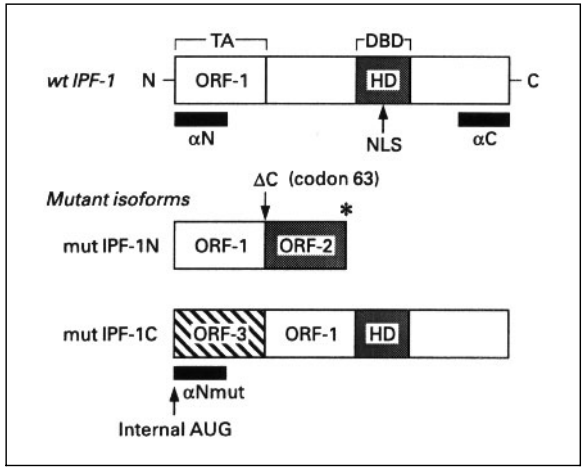

Fig. 2. Pro63fsdelC mutation in IPF-1 causes haploinsufficiency and possible dominant negative inhibition of IPF-1 functions in MODY4. A cytosine deletion (ΔC) at codon 63 in the coding sequence of IPF-1 (Open Reading Frame ORF-1) results in a translational frameshift (ORF-2), premature termination of translation (asterisk), and initiation of internal translation (internal AUG) of a dominant negative inhibitor protein. Note that the internal translation initiates translation in ORF-3 and switches back to the wild-type (wt) translational frame (ORF-1) when encountering the cytosine deletion (ΔC) in codon 63. The mutant IPF-1N isoform consists of the N-terminal transactivation domain (TA) devoid of the DNA-binding domain (DBD) and included nuclear translocation signal (NLS). The mutant IPF-1C consists of the DNA-binding homeodomain lacking the transactivation domain encoded by ORF-1 (ORF-3 does not encode transactivational sequences). The αN, αC, and αN Mut designate antisera made to small peptides encoded by ORF-1 (αN, αC) or ORF-3 (αN mut) [1].

of the extended family revealed a high prevalence of diabetes of early onset, <40 years of age and a strong Mendelian inheritance. An initial linkage analysis in 27 family members of diabetes with heterozygosity for the Pro63fsdelC mutation in *ipf*-1 gave a highly significant Lod score of 3.43 [2]. A subsequent more extensive analysis of this family encompassing more than 90 members uncovered 21 members with diagnosed diabetes or glucose intolerance by either testing or reported history, five of whom have a documented onset of diabetes or glucose intolerance before the age of 30. MODY is defined as a familial form of type 2 diabetes with very early onset (childhood, adolescence, early adulthood) and autosomal dominant inheritance associated with defects in insulin secretion [6]. Thus the family carrying the Pro63fsdelC mutation is clearly a MODY family, designated MODY4.

Insulin secretion rates (ISR) and glucose utilization were determined in 15 members of the family (7NM, 8NN, in which N = normal wild-type allele

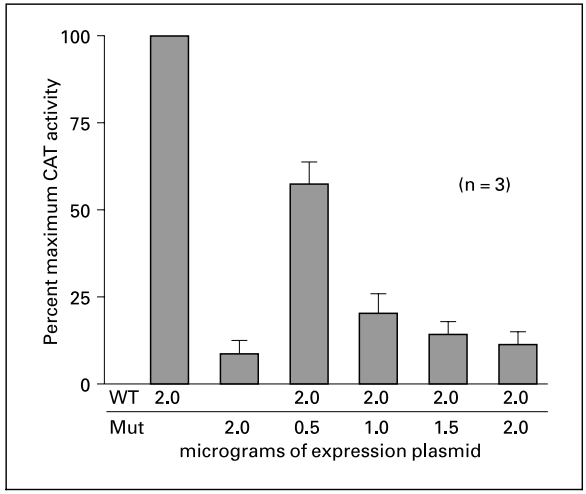

Fig. 3. Dominant negative inhibition of transactivation of gene transcription by IPF-1 with the inactivation Pro63fsdelC mutation (mut). HeLa cells were transfected with a CAT (chloramphenicol acetyltransferase) transcriptional reporter containing five copies of the upstream enhancer (A3/E2, far flat) and expression plasmids encoding the basic helix-loop-helix transcription factorE47, wild-type IPF-1 (wt) or increasing amounts of the mutant IPF-1. Increase in amounts of IPF-1 mut inhibit transactivation of the A3/E2 reporter by IPF-1 wt.

and M = mutant allele) using a five-step glucose clamp protocol followed by a 30 minute infusion of the insulinotropic hormone, glucagon-like peptide-1 (GLP-1). The 7 individuals carrying the *ipf*-1 mutation have severely impaired insulin secretion compared to the 8 unaffected members [25]. In addition, glucose utilization by NM individuals is significantly higher than that of NN individuals indicating that the less obese individuals with the *ipf*-1 mutation (NMs) are more insulin sensitive compared to the normal (NN) more obese family members.

Mutations in the *ipf*-1 Gene Associated with Common Type 2 Diabetes

The discovery of a mutation in the *ipf*-1 gene prompted a search for *ipf*-1 mutations in diabetic individuals in a large population in which familial diabetes has been identified, the French population. A screening of members of 192 French families with a strong familial history of diabetes identified three additional *ipf*-1 mutations associated with type 2 diabetes (fig. 4). Although the

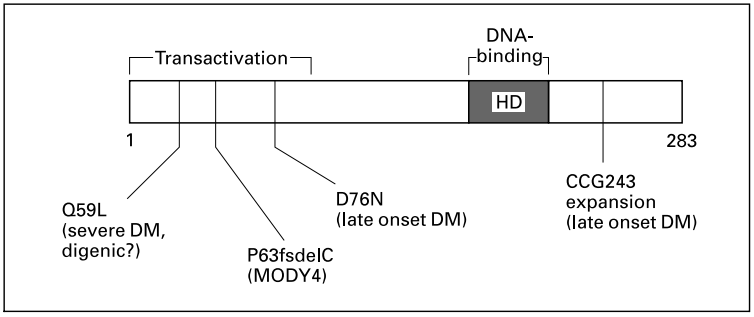

Fig. 4. Mutations in IPF-1 associated with late onset type 2 diabetes. Two missense mutations (Q59L and D76N) and one CCG codon expansion mutation (InsCCG243) identified by screening of families with late onset type diabetes in the French population. The Q59L and D76N mutations are transmitted in linkage disequilibrium with diabetes. The InsCCG243 mutation is linked to late onset diabetes in two extended families. Also shown is the P63fsdelC mutation present in the family with MODY4.

Pro63fsdelC mutation (MODY4) was not found in this screen, two missense mutations, Q59L (one family) and D76N (nine families), and a proline insertion due to what may be an early trinucleotide expansion, InsCCG243 (two families) were uncovered. Notably, these three *ipf*-1 mutations are associated with the more common late onset type 2 diabetes. Collectively, these three *ipf*-1 mutations account by extrapolation for 6.2% of type 2 diabetes in the French population. Additional mutations in *ipf*-1 associated with type 2 diabetes have been identified in screenings of UK, Danish, and Finnish populations. Thus far several different *ipf*-1 mutations are recognized to be associated with diabetes. The D76N mutation appears to be particularly prevalent in the French (4.7%) and UK (2.4%) families who manifest common type 2 diabetes.

The D76N and Q59L missense mutations both occur in the known transactivation region of IPF-1 located aminoproximal to the homeodomain. The CCG insertion mutation, Ins CCG243, occurs in a cryptic transactivation domain that includes six prolines encoded by a stretch of four CCG and two CCC codons, thereby providing a sixth proline in the sequence [26]. It is worth noting that mutations in codons encoding polyproline tracts occur also in the transcription factors HNF-1α (MODY3) and HNF-4α (MODY1). For example, a common mutation in HNF-1α is an insertion of a cytosine in a tract encoding polyprolines (Pro291fsinsC) [27]. Deletions or insertions of a nucleotide in the protein coding sequence of a gene causes a shift in the translational reading frame inevitably resulting in a premature termination of translation and the formation of a foreshortened, truncated protein. Such

mutations are by definition inactivating mutations resulting in hemizygosity and haploinsufficiency for the functional protein. One explanation for the relatively high frequency of occurrence or such mutations in DNA sequences encoding polyproline tracts is that the codons for proline (CCX) are enriched in cytosines. It is well known that the fidelity of DNA polymerase can be poor when encountering polypyrimidine tracts, resulting in the insertion or deletion of a pyrimidine base pair (cytosine or thymidine) [28, 29].

Digenic Causations of Type 2 Diabetes

As the numbers of identified mutations in specific genes (e.g. *ipf*-1, *hnf*-1α, *hnf*-1β, *hnf*-4α, *gk*) increases, the probability of coinheritance of two different mutations (compound heterozygosity) also increases. We propose that such a circumstance be designated as digenic diabetes (Gr di (two), genic (genesis, origin)). One example has been discovered of coinheritance of mutations in *ipf*-1 and *ib*-1 (islet brain-1) genes. Heterozygosity for a missense mutation (S59N) in the transcription factor IB-1 results in late onset diabetes. Likewise, heterozygosity for the Q59L mutation in IPF-1 is associated with a late onset diabetes. However, one family with the Q59L IPF-1 mutation also carried the S59N IB-1 mutation and subjects who inherit both the IPF-1 and the IB-1 mutation manifest a severe, early onset form of diabetes. Several additional families are currently under investigation who have a high prevalence of late onset diabetes in which coinheritance of defective HNF-1α and IPF-1 alleles are suspected to be responsible for the causation of type 2 diabetes.

It is estimated that mutations in the now recognized diabetes genes (*ipf*-1, *hnf*-1α, *hnf*-4α, *hnf*-1β, *Ib*-1, *gk*) may account for 10–15% of all type 2 diabetes. As a consequence of gene knockout experiments in mice that result in a phenotype of defective β-cell development, it is tempting to speculate that mutations in genes encoding the transcription factors Isl-1, Beta2/NeuroD, Pax6, Pax4, Nkx 2.2, Nkx 6.1, may also contribute to the genetic causes of type 2 diabetes (table 2).

Summary

The prevalence of type 2 diabetes mellitus is increasing in populations of the world. The causation of type 2 diabetes remains poorly understood and is generally believed to be a polygenic disorder with complex inheritance modified by environmental factors. However, mutations in several specific genes have now been identified as responsible for a subset of monogenic type

Table 2. Transcription factors in which targeted gene disruption in mice results in defective pancreas development

Factor	Knockout phenotype
Homeodomain proteins	
IDX-1	Pancreatic agenesis, epithelial cell
Pax-4	β and δ-cell agenesis α-cell hyperplasia
Pax-6	α-cell hypogenesis
Isl-1	Islet agenesis, absent dorsal mesenchyme
Nkx2.2	β-cell aplasia, arrested development
Nkx6.1	
bHLH proteins	
E2A (E47)	No phenotype
Beta-2	β-cell agenesis

Nullizygosity for IDX-1 (IPF-1/PDX-1) results in pancreatic agenesis [22]. Pax-4 null mice [30] and Nkx2.2 null mice [31] manifest a selective severe impairment of β-cell development with α-cells expanded or unaffected. Disruption of the Pax-6 gene impairs α-cell and β-cell development but impairment of α-cell development is more severe [32, 33]. Isl-1 null mice have a highly selective failure of early development of the dorsal pancreas leaving the ventral pancreas unaffected, but the mutation is embryonic lethal at embryonic day 95, thereby precluding further temporal and spatial analyses of pancreas development [34]. The knockout of the Beta2/NeuroD gene results in β-cell agenesis/hypogenesis with a marked reduction in β-cell mass and failure of the development of organized islets [35]. Notably, the mice nullizygous for the E2A gene encoding the bHLH transcription factor E47, related to Beta2/NeuroD apparently have no detectable pancreatic phenotype [36].

2 diabetes generally described as MODY, maturity onset of diabetes of the young, but now also recognized to be a cause of common, late onset type 2 diabetes. Five of the six known genes responsible for MODY encode transcription factors important in β-cell functioning.

One of the transcription factors, IPF-1 (IDX-1/PDX-1/STF-1) illustrates the emerging understanding that the inheritance of type 2 diabetes may not be as complex as previously supposed. For example, different mutations in

the *ipf*-1 gene present as either MODY, or are strong genetic components of early onset, or late onset diabetes, depending on the nature of the mutation. An inactivating mutation in the *ipf*-1 gene, Pro63fsdelC, that results in haploinsufficiency and possibly a dominant negative effect (1) causes MODY in heterozygous individuals (2) and pancreatic agenesis in the homozygous condition [3]. Somewhat less severe mutations of *ipf*-1 such as several missense mutations or a CCG trinucleotide expansion correlate with, are associated with, or are susceptibility factors for a phenotype of late onset diabetes. At this time, various mutations in the *ipf*-1 gene alone may account for 4–6% of all type 2 diabetes in the French and U.K. populations. Collectively, mutations in the genes encoding IPF-1, HNF-1α, HNF-4α, HNF-1β and glucokinase may be responsible for a substantial subset of type 2 diabetes worldwide.

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Joel F. Habener, MD, Laboratory of Molecular Endocrinology, Massachusetts General Hospital, 55 Fruit Street, WEL320, Boston, MA 02114 (USA)
Tel. +1 617 726 5190, Fax +1 617 726 6954, E-Mail jhabener@partners.org

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IRS-2 Is a Common Element in Peripheral Insulin Signaling and β-Cell Function

Morris White

Howard Hughes Medical Institute, Joslin Diabetes Center, Harvard Medical School, Boston, Mass., USA

The integration of multiple signals to coordinate cellular growth and metabolism, initiate differentiation, and prolong the survival of cells is a complicated biological problem with significant medical relevance. These mechanisms are especially important during development and maintenance of the nervous system, communication between cells of the immune system, evolution of transformed cells and metabolic control. Growing evidence suggests that noninsulin-dependent diabetes mellitus (type 2 diabetes) is due to defects in insulin action that are exacerbated by reduced insulin secretion (fig. 1). Moreover, recently work with mice lacking the gene for IRS-2, one of the insulin receptor substrates, suggest that signaling pathways used in peripheral tissues to mediate the insulin response may also be operational in pancreatic islets to promote growth or survival of β-cells [1]. Thus, rational treatments for type 2 diabetes would ultimately include new drugs that modify common signaling pathways that fail in the peripheral tissues and β-cells.

Overview of the Insulin Signaling System

The insulin receptor is a tyrosine kinase, which initiates a cascade of biological responses following insulin binding. Tyrosine kinase coupled receptors play broad roles during signal transduction by many cytokines and growth factors. Growth factor and cytokine receptors are generally composed of extracellular ligand-binding domains and intracellular tyrosine kinases, or domains that recruit and regulate cytoplasmic tyrosine kinases; these receptors

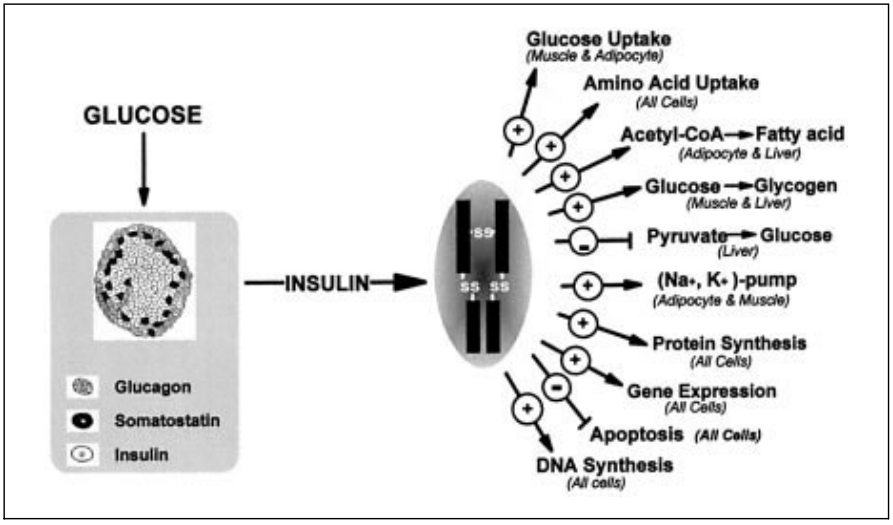

Fig. 1. A diagram of the pleiotropic response to insulin secreted from glucose-stimulated pancreatic β-cells.

are broadly organized into various classes based on structure. Although tyrosine kinase receptors are structurally diverse, ligand-stimulated dimerization is commonly employed to activate these signaling pathways [2].

The insulin receptor and the related insulin-like growth factor-1 receptor are unusual as they are heterotetramers composed of two covalently linked extracellular α-subunits each of which are linked to transmembrane β-subunits (fig. 2). Thee α-subunits form the insulin-binding domain, whereas the β-subunits contained the tyrosine kinase activity. Since insulin receptors are covalent dimers, activation of the kinase probably involves an alteration of the α-subunit dimer during insulin binding. Following insulin binding, the several regions in the β-subunit becomes tyrosine phosphorylated which increase specific activity of the enzyme. In many cases, autophosphorylation alone is adequate to mediate biological signals through direct interaction with signaling proteins [3]; however, the insulin and IGF-1 receptors' additional docking proteins are phosphorylated by the activated kinase, which provide the link to various signaling proteins.

The identification of substrates for the insulin receptor progressed slowly in the beginning because these proteins are difficult to identify, purify and clone. In the meantime, the notion that insulin receptor autophosphorylation was sufficient for signaling gained popularity. This model was supported by studies on other growth factor receptors, which were found to bind directly and selectively to the Src homology 2 domains in various enzymes and adapter

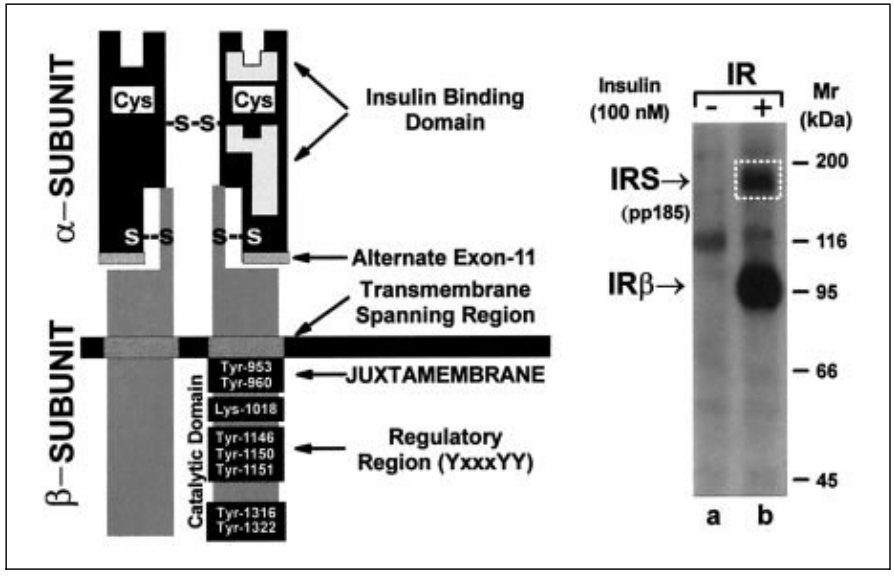

Fig. 2. A schematic representation of the insulin receptor. Several important structural elements are illustrated.

molecules (SH2-proteins) [3–5]. However, it continues to be difficult to show direct interactions between the insulin receptor and SH2-proteins, although the few examples are now available [6–10]. By contrast, autophosphorylation of the insulin receptor β-subunit strongly activates the tyrosine kinase, a characteristic that is not easy to demonstrate for many receptor kinases [11–13]. The activated insulin receptor phosphorylates several docking proteins on tyrosine residues, which bind to the SH2 domain in various signaling proteins (SH2-proteins) [14, 15]. The first evidence supporting this indirect mechanism was obtained over 10 years ago with the identification of pp185 [16]. Today, at least 6 related proteins have been purified and cloned, including IRS-1, -2, -3 and IRS-4, and Gab-1 and $p62^{dok}$ [16, 17–20]. Each of these proteins are composed of conserved NH_2-terminal domains that couple the substrate to the activated insulin receptor, and multiple tyrosine phosphorylation sites in COOH-terminus that bind various SH2-proteins (fig. 3).

The IRS-Protein Signaling System

The discovery of IRS-1 provided the first example of a cytoplasmic docking protein coupling an activated receptor tyrosine kinases to the Src homology

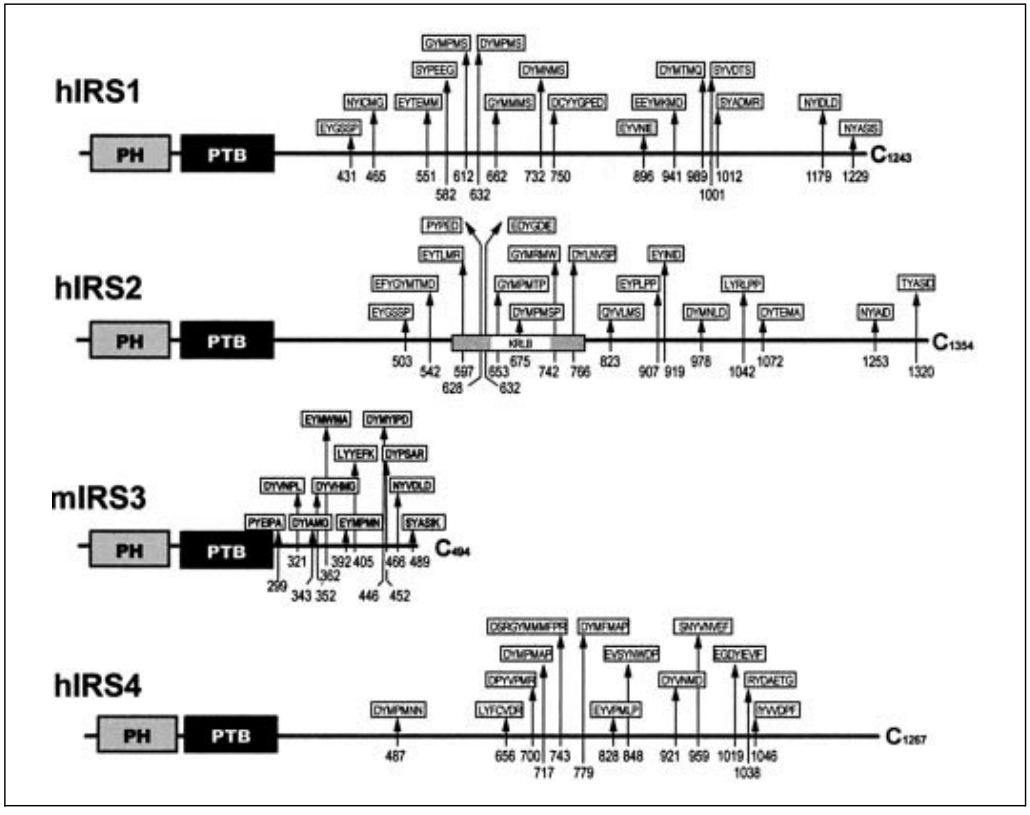

Fig. 3. A schematic comparison of functional elements in IRS-1, IRS-2, IRS-3 and IRS-4. The relative position of the pleckstrin homology (PH) and phosphotyrosine binding (PTB) domain are indicated. Phosphorylation sites expected to bind p85, Grb-2 or SHP2 are indicated; the relative positions of other putative phosphorylation sites are indicated.

2 domain in various signaling proteins [16]. The importance of IRS-proteins for insulin signaling was first suggested by studies using cells expressing a mutant insulin receptor lacking Tyr_{960} in the juxtamembrane region [21]. In these cells, the endogenous substrate(s) of the receptor were not phosphorylated despite a 'normal activation' of receptor autophosphorylation. Several biological responses to insulin were also impaired. Thus, these results were consistent with the notion that phosphorylation of IRS-proteins (called pp185 at that time), not just autophosphorylation, mediated the biological response to insulin.

The subsequent purification of pp185 and the cloning of IRS-1 provided clear evidence of intracellular substrates of the insulin receptor [14, 16, 22].

The use of recombinant IRS-1 to rescue insulin signaling in myeloid progenitor cells which lack IRS-proteins (IRS-proteins) provided the first direct evidence that IRS-1 and related substrates are important elements for insulin signaling [23]. In the same study, IRS-1 was shown to mediate IL4 signaling, which lead directly to the discovery that IRS-proteins are involved in other signal transduction cascades, a concept that has now been extended to include several interleukins, growth hormone, prolactin and interferons [15].

IRS-proteins provide important flexibility during receptor signaling. First, they amplify receptor signaling by eliminating the stoichiometric constraints encountered by receptors that directly recruit SH2-proteins to their autophosphorylation sites. Moreover, IRS-proteins dissociate the intracellular signaling complex from the endocytic fate of the activated receptor. This feature may be especially important for insulin-stimulated biological effects such as glucose uptake, which involves signal transmission to membrane compartments that are inaccessible to the insulin receptor [24]. The ability of a single receptor to engage multiple IRS-protein further expands the repertoire of signaling pathways that can be regulated. Finally, a single IRS-protein can interact with multiple receptors, including members of the IL-2 and IL-6 receptor family, as well as receptors that mediate inhibitory signals such as TNFα [25, 26]. This framework provides a molecular basis to understand the relation between distinct signaling pathways that may contribute to the complications encountered by diabetic individuals.

The assembly of multi-component signaling complexes is a common mechanism for signal transduction by growth factor and cytokine receptors with intrinsic or associated tyrosine kinase activity. In many cases, the Src homology-2 domains in various signaling proteins (SH2-proteins) bind directly to phosphotyrosine residues in the activated receptors. A growing number of receptors utilize intermediate docking proteins to engage and regulated SH-protein. Several years ago, we isolated a cDNA clone for IRS-1, the prototype for a new class of docking proteins that are phosphorylated on multiple tyrosine residues during transient association with the activated receptors for insulin, IGF-1, IL-4 and other cytokines.

Recently, several closely related IRS-proteins were identified, including IRS-2, IRS-3 and IRS-4. Comparison of their amino acid sequences reveals several common features, including a well-conserved pleckstrin homology (PH) domain at the extreme NH_2-terminus, followed immediately by a phosphotyrosine-binding (BTB) domain that binds to phosphorylated NPXY-motifs (fig. 2). However, the COOH-terminal regions of these proteins are similar only in that they contain multiple tyrosine phosphorylation motifs. IRS-1 binds several SH2-proteins, including p85, Grb-2, SHP2, nck, crk, fyn and others (fig. 2) [27–31]. However, the comparative association of IRS-1 and

IRS-2 with recombinant SH2-domains suggests overlapping and distinct signaling potential [32]. Interestingly, immortalized fibroblasts lacking IRS-1 while overexpressing IRS-2 display responses to insulin that are distinct from those observed in fibroblasts overexpressing IRS-1 [33]. Moreover, the striking physiological differences in mice lacking IRS-1 or IRS-2 amply confirm this hypothesis [1].

The most recent additions to the IRS-protein family, IRS-3 and IRS-4, contain highly conserved PH and PTB domains (fig. 3). Unlike IRS-1 and IRS-2 the expression of these proteins is largely restricted to adipocytes and pituitary/thymus, respectively. The COOH-termini of the IRS-3 and IRS-4 are the least similar to IRS-1 and IRS-2. IRS-3 has the shortest COOH-terminus. It contains several binding sites for p85 and two sites in the COOH-terminus expected to bind SHP2 [18]; IRS-3 is a strong activator of the PI-3 kinase [34]. By contrast, IRS-4 does not contain sites expected to bind SHP2, but contains four motifs that are expected to bind p85 [19]; however, IRS-4 is a poor activator of PI 3-kinase (Uchida and White, in preparation). This result is unexpected, especially as the absence of SHP2-binding sites is expected to enhance the phosphorylation of the YXXM-motifs that recruit p85. Thus, IRS-proteins are not redundant pathways, but provide distinct elements that contribute to the pleiotropic insulin response.

IRS-2 and Type 2 Diabetes

Diabetes mellitus results from the absence of insulin (type 1 diabetes), or uncompensated peripheral insulin resistance owing to relative insulin deficiency (type 2 diabetes). Type 2 diabetes mellitus accounts for $>90\%$ of the cases. The insulin receptor was originally thought to be involved, but mutations at this locus are rare and when present they are associated with very severe insulin resistance; diabetes does not always occur owing to the occurrence of compensatory hyperinsulinemia [35, 36]. Common type 2 diabetes is characterized by impaired insulin-stimulated glucose uptake into skeletal muscle and adipocytes; impaired inhibition by insulin of hepatic gluconeogenesis; and dysregulated insulin secretion. Susceptibility to both insulin resistance and insulin secretory defects appears to be genetically determined. Environmental factors, especially diet, physical activity, and age, interact with genetic predisposition to affect disease prevalence [37]. Defects in insulin action precede the overt disease and are seen in nondiabetic relatives of diabetic subjects. However, insulin resistance alone does not ordinarily cause type 2 diabetes: As β-cells fail to compensate over time, even mild insulin resistance contributes significantly to severe type 2 diabetes.

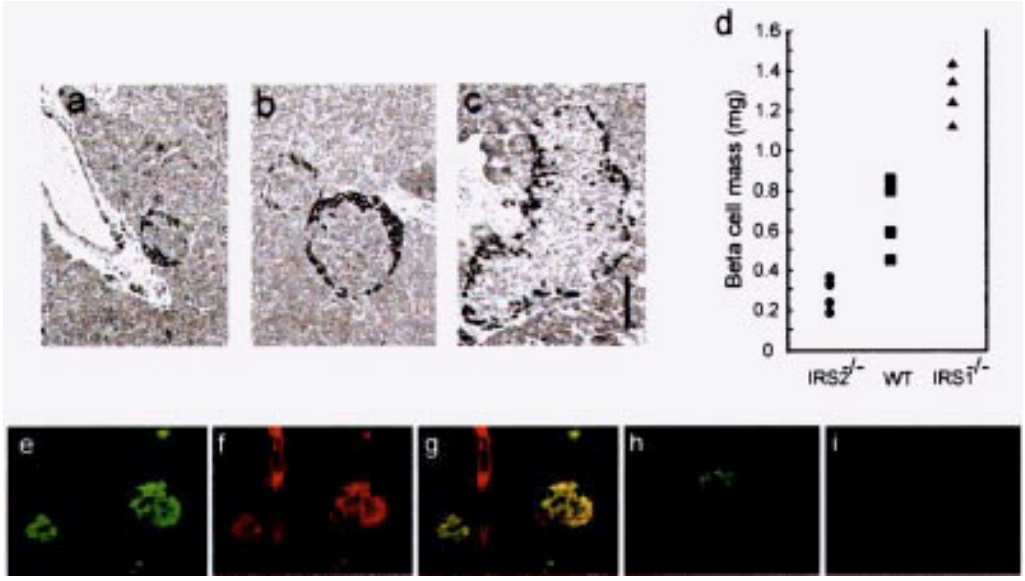

Fig. 4. Islet morphology and analysis of β-cell mass in IRS2$^{-/-}$ mice compared to IRS1$^{-/-}$ and wild-type mice and expression of IRS-2 in islets of wild-type and IRS2$^{-/-}$ mice. Representative islets from (*a*) IRS2$^{-/-}$, (*b*) wild-type, and (*c*) IRS1$^{-/-}$, animals are shown. Quantification of β-cell mass by point-counting morphometric analysis (*d*); immunostaining in wild-type animals for insulin (*e*); IRS-2 (*f*) and colocalization (*g*). In IRS2$^{-/-}$ animals, immunostaining for insulin (*h*) and IRS-2 (*i*).

β-cell dysfunction alone, owing to mutation in glucokinase or various transcription factors (HNF-1α, HNF-4α) can cause mild type 2 diabetes [38–41]. However, these mutations are found in $< 5\%$ of the patients diagnosed with type 2 diabetes before the age of 40, and do not directly cause insulin resistance which typically proceeds the occurrence of common type diabetes. A molecular basis for the common forms of type 2 diabetes remain unknown, but it should be expected to involve disruption of both peripheral insulin action and β-cell compensation.

The IRS-proteins established an attractive focus to explore the molecular basis of diabetes. However, our original results were not encouraging: IRS-1-deficient mice display growth retardation (due to IFG-1 resistance) and impaired glucose tolerance (due to insulin resistance), but never develop diabetes [42]. Recently, we demonstrated that type 2 diabetes develops in mice lacking the *irs2* gene product [1]. Since insulin resistance alone is not sufficient to cause diabetes in people or mice, this result suggests that IRS-2 is also important for β-cell function. In the early stages of the development of diabetes in the IRS-2

knockout mice, glucose-stimulated insulin release in vivo is normal, and before weaning modest hyperinsulinemia apparently compensates for insulin resistance. However, the failure to expand β-cell mass after weaning apparently results in hyperglycemia because compensatory insulin release is attenuated (fig. 4). However, the role of IRS-2 in insulin secretion is unknown, although it could involve regulation of certain transcription factors required for insulin synthesis. This sequence is reminiscent of type 2 diabetes in humans and may result, at least in part from 'glucose toxicity' or glucose-induced desensitization. The presence of IRS-2 in pancreatic ductal epithelium, the site of neogenesis highlights a direct role for IRS-2-dependent signaling in the regulation of islet cell replication, neogenesis and/or apoptosis.

It is now possible to hypothesize that dysfunction of one signaling pathway may underlie multiple defects in growth, glucose homeostasis, and diabetes. The discovery that IRS-2 mediates peripheral insulin signaling and β-cell survival provides the best example thus far of how a single genetic defect might cause the complex pathogenesis of type 2 diabetes. The development of type 2 diabetes in $IRS2^{-/-}$ mice provides new insight into the role of β-cell compensation in the pathogenesis of this disease. Our findings suggest that IRS-2 is a critical regulator of β-cell survival or growth. Thus, IRS-2 may represent an appropriate target for rationale drug design, and a full characterization of the role of IRS-2 in β-cell function could advance this therapeutic aim.

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Morris F. White, PhD, Howard Hughes Medical Institute, Joslin Diabetes Center,
1 Joslin Place, Boston, MA 02215 (USA)
Tel. +1 617 732 2578, Fax +1 617 732 2593, E-Mail Morris.White@joslin.harvard.edu

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Regulation of Visceral Endoderm Differentiation and Gene Expression by the MODY1 Transcription Factor HNF-4α

Stephen A. Duncan

Department of Cell Biology, Neurobiology and Anatomy, Medical College of Wisconsin, Milwaukee, Wisc., USA

Development of a simple two-cell embryo to a complex multicellular organism is a highly dynamic procedure requiring orchestrated cell movements and multiple interactions between cells and their surroundings. The result of these intricate processes is the controlled differentiation of populations of progenitor cells to produce novel cell lineages. The phenotype of a given cell type is determined by the gamut of genes it expresses and this is regulated, in a large part, at the level of transcription [1]. In order to understand the molecular mechanisms underlying development of a given cell type, tissue or organ it is, therefore, crucial to identify the transcription factors which ultimately control the process. Unfortunately, because most organs are a complex array of different cell types and tissues, all of which dynamically interact to regulate organogenesis, it can be difficult to measure the contribution of a specific transcription factor to overall organ development. However, the liver offers a relatively less complicated system to study transcriptional regulation of development because over 90% of its cells are hepatocytes.

Hepatocyte Nuclear Factor 4α (HNF-4α)

Numerous approaches have been used to identify transcription factors with potentially important roles in mammalian development [2–5]. One way is to identify transcription factors which regulate gene expression in a given adult tissue and subsequently determine whether they have roles in regulating embryonic development of the corresponding fetal tissue. With the purpose

of identifying transcription factors which may regulate liver development the Darnell laboratory undertook a molecular dissection of promoters controlling hepatocyte expression of α-1 antitrypsin, transthyretin, and apolipoprotein-CIII genes [6–9]. During these analyses they identified a transcription factor called hepatocyte nuclear factor (HNF)-4α (originally called HNF-4) [9].

HNF-4α is a zinc finger transcription factor and a member of the steroid hormone receptor family [9]. Although originally classified as an orphan receptor it has recently been shown that fatty acyl-CoA thioesters are ligands which can effect HNF-4α activity [9, 10]. Detailed structure/function analyses have shown that HNF-4α has domains mediating transcriptional activation, ligand binding, dimerization, and DNA binding [9, 11–13]. HNF-4α is also phosphorylated on serine/threonine and tyrosine residues and this appears to be important for its activity [14, 15]. Although HNF-4α was found to bind to elements within the promoters of many gene expressed in the liver, expression of HNF-4α itself was not restricted to the liver. In the adult rodent, HNF-4α mRNA was detected in the liver, kidney, and intestine but was absent from many other tissues including spleen, lung and heart [9].

HNF-4α Is Expressed in the Embryonic Liver and Extraembryonic Visceral Endoderm

Several lines of evidence suggested that HNF-4α could act as a regulator of hepatic differentiation: (i) In somatic cell hybrids, expression of hepatic marker genes is extinguished by the presence of specific 'extinguisher loci'. However, the ability of hybrids to reexpress hepatocyte genes, *en bloc*, correlates with expression of the HNF-4α but not with other liver transcription factors [16]. Similar results were reported using hepatoma cell variants to correlate HNF-4α with expression of multiple liver genes [17]. (ii) HNF-4α was found to be capable of transactivating expression from the HNF-1α promoter [18, 19]. In addition, forced expression of HNF-4α in a dedifferentiated hepatoma cell line induced expression of HNF-1α [19]. This suggested the existence of a transcription factor hierarchy with HNF-4α acting upstream. (iii) Finally, a chromosomal deletion encompassing the *drosophila* homologue of HNF-4α (dHNF-4) results in severe defects in midgut and malpighian tubule development [20]. Cumulatively these results suggest that HNF-4α, either acting alone or by activating expression of HNF-1α, could control development of the liver.

The individual stages of liver development have been reviewed in detail by K. Zaret [21]. Briefly, the hepatocytes and biliary epithelium of the liver derive from a ventral portion of the foregut endoderm. Between 8.0–8.25 days

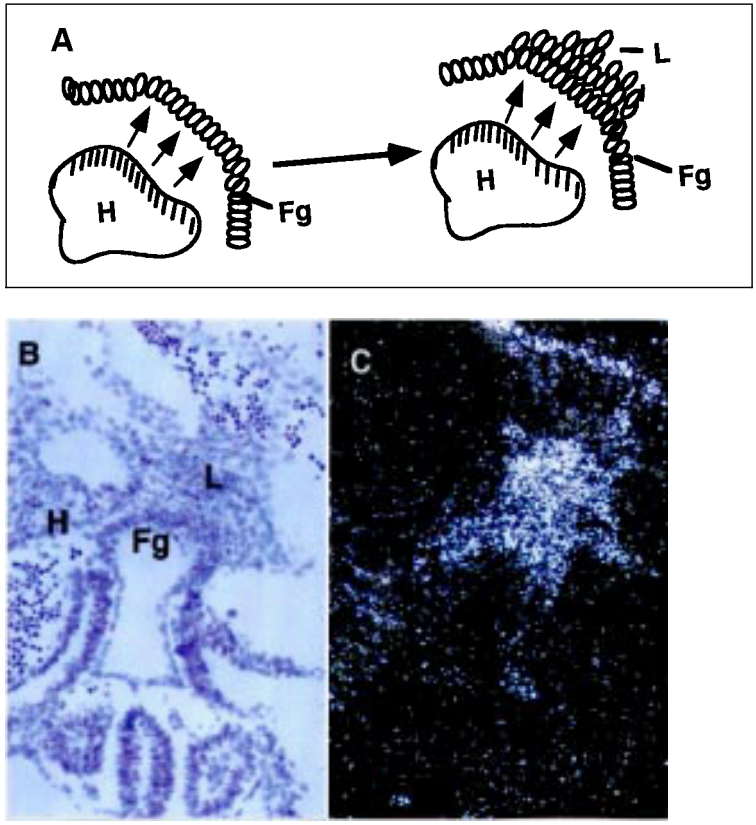


Fig. 1. HNF-4α is expressed in the developing liver primordium. *A* Shows a schematic representation of early stages of liver development. A secreted signal from the developing cardiac mesoderm (H) induces proliferation a portion of foregut endoderm (Fg) which will subsequently follow a hepatic fate (L). *B* and *C* In situ hybridization showing expression of HNF-4α mRNA in the liver primordium. In situ hybridization was performed on a transverse section through the developing liver of a mouse embryo at approximately 8.5 days gestation. Sections were hybridized to a radiolabeled antisense HNF-4α probe. *B* Shows a phase contrast micrograph of a hematoxylin and eosin stained section and the corresponding dark-field is shown in *C*. In *C* HNF-4α mRNA can be visualized by the presence of silver grains after emulsion autoradiography (white staining) [26]. Figure adapted from Duncan et al. [27].

of gestation the embryonic endoderm of the foregut invaginates inwards such that a portion of it, called the hepatic field, lies adjacent to the developing heart. As shown in figure 1a, at around 8.5 days of gestation developing cardiac mesoderm induces cells of the hepatic field to proliferate and differentiate [22–24]. As the endoderm proliferates endoderm cells of the hepatic

field migrate outward to interact with the mesenchyme of the septum transversum. During this time the prehepatic cells differentiate and begin to express a large number of genes associated with liver function. If HNF-4α did indeed have a role in hepatic differentiation it should be expressed in the liver during embryogenesis. This was tested by mapping the tissue expression pattern of HNF-4α mRNA during mouse development by radioactive in situ hybridization [25, 26]. Figure 1b and c shows that the first fetal expression of HNF-4α mRNA was detected in the developing liver primordia and hindgut at around 8.5 days of gestation [27, 28]. During midgestation stages of development HNF-4α mRNA was found in the developing intestine, rectum, pancreas, liver, kidney and stomach [27, 28]. This fetal expression of HNF-4α mRNA throughout midgestational stages of development is similar to that seen in the adult.

In addition to expression in these developing fetal tissues, HNF-4α mRNA was found in the extraembryonic visceral endoderm throughout postimplantation stages of development [27, 28]. The extraembryonic visceral endoderm is distinct from the embryonic endoderm that forms the liver. The visceral endoderm never forms part of the fetus; instead it contributes the endodermal component of the yolk sac. Lineage tracing experiments have shown that the visceral endoderm derives from the primitive endoderm which is formed during implantation [29]. Figure 2 shows that HNF-4α could be detected in the primitive endoderm as early as 4.5 days of gestation [27]. At this time in development the primitive endoderm forms a distinct epithelial layer of cells atop the inner-cell mass. As is the case for the hepatic primordia, HNF-4α expressed during the onset of extraembryonic endoderm development.

HNNF-4α Expression in the Extraembryonic Visceral Endoderm Is Essential for Gastrulation

The expression of HNF-4α mRNA during the genesis of both hepatic and visceral endoderm suggested a role for HNF-4α in regulating multiple early developmental processes. To test this Chen et al. generated HNF-4α –/– mouse embryos by gene targeting in embryonic stem (ES cells) [30]. The first indication that HNF-4α was crucial for embryogenesis was provided by the finding that no HNF-4α –/– offspring could be recovered from the breeding of HNF-4α +/– mice. Subsequent morphological and histological characterization of HNF-4α –/– embryos revealed that their development arrested prior to the onset of hepatogenesis. As shown in figure 3, HNF-4α –/– embryos were grossly abnormal by 7.5 days gestation due to severe defects in gastrulation. While wild-type embryos showed formation of the primitive streak

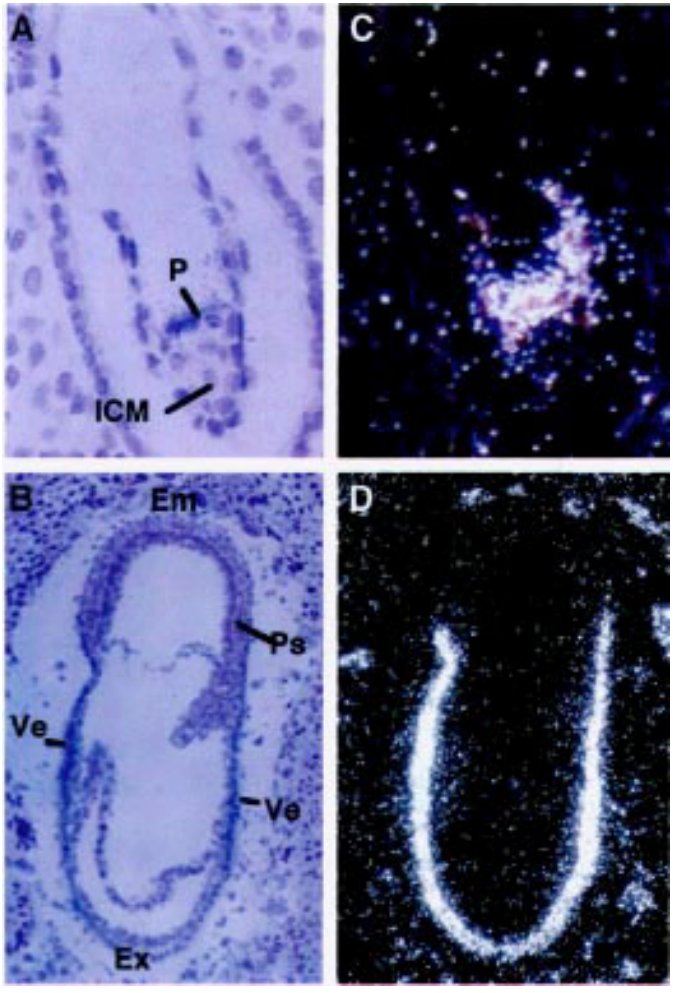


Fig. 2. HNF-4α is expressed in the extraembryonic primitive and visceral endoderms. In situ hybridizations were performed on sections through mouse embryos at 4.5 (*A* and *C*) and 7.5 (*B* and *D*) days gestation using radiolabeled antisense HNF-4α probes. Phase contrast micrographs are shown in *A* and *B* and corresponding dark field images in *C* and *D*. P = Primitive endoderm; ICM = inner cell mass; Em = embryonic pole; Ex = extraembryonic pole; Ve = visceral endoderm; Ps = primitive streak. Figure adapted from Duncan et al. [27].

and compartmentalization due to formation of the chorion and amnion, mutant embryos exhibited no signs of these distinctive morphological features. Gastrulation is a critical embryonic process during which mesodermal cells are produced and the entire body plan is set. Disruption to this process, therefore, results in embryonic lethality. Examination of marker gene expres-

sion by in situ hybridization revealed that initiation of gastrulation was delayed in HNF-4α –/– embryos. This can been seen in figure 3 which compares expression of the brachyury gene in HNF-4α +/– and HNF-4α –/– embryos at 7.5 days gestation. In wild-type embryos brachyury is highly expressed in the nascent mesoderm which forms at the primitive streak. This expression of brachyury in the primitive streak is characteristic of a wildtype midgastrulation stage embryo. In contrast, figure 3 shows expression of brachyury is limited to a few cells of embryonic ectoderm present in the developing HNF-4α –/– embryo. In these embryos gastrulation appeared to arrest shortly after its onset, resulting in the absence of fully differentiated mesoderm [30]. In addition, HNF-4α –/– embryos exhibited a significant increase in apoptotic cell death [30, 31].

From the above data it was concluded that HNF-4α was essential for gastrulation [30]. As shown in figure 1, between 4.5 and 8.5 days gestation the only expression of HNF-4α detectable by in situ hybridization was in the extraembryonic visceral endoderm [27]. This strongly suggested that gastrulation in the mouse required a paracrine function of the visceral endoderm that was regulated by HNF-4α. For this to be correct two criteria should be fulfilled: (i) that expression of secreted proteins should be dysregulated in an HNF-4α –/– visceral endoderm, and (ii) that complementation of HNF-4α –/– embryos with a wild-type visceral endoderm should allow HNF-4α –/– embryos to complete gastrulation.

HNF-4α Is an Extraembryonic Visceral Endoderm Differentiation Factor

Expression of secreted serum factors from the visceral endoderm could be disrupted either by a block in visceral endoderm specification (formation) or by a dysregulation of target gene expression. It, therefore seemed pertinent to determine if the visceral endoderm could properly differentiate in the absence of HNF-4α. The first steps in differentiation of visceral endoderm occur at 4.5 days of gestation just as the blastocyst implants. The small size of such early postimplantation embryos makes it difficult to carry out quantitative molecular analyses. However, an alternative approach was provided by the ability of ES cell embryoid bodies to form visceral endoderm in vitro. If ES cells are cultured in suspension in the absence of leukemia inhibitory factor (LIF) they differentiate to form embryoid bodies which contain an outer layer of visceral endoderm. As was the case in the embryo, in situ hybridization analyses showed that expression of HNF-4α mRNA was restricted to the visceral endoderm of day 14 ES cell embryoid bodies [25]. To analyze the role

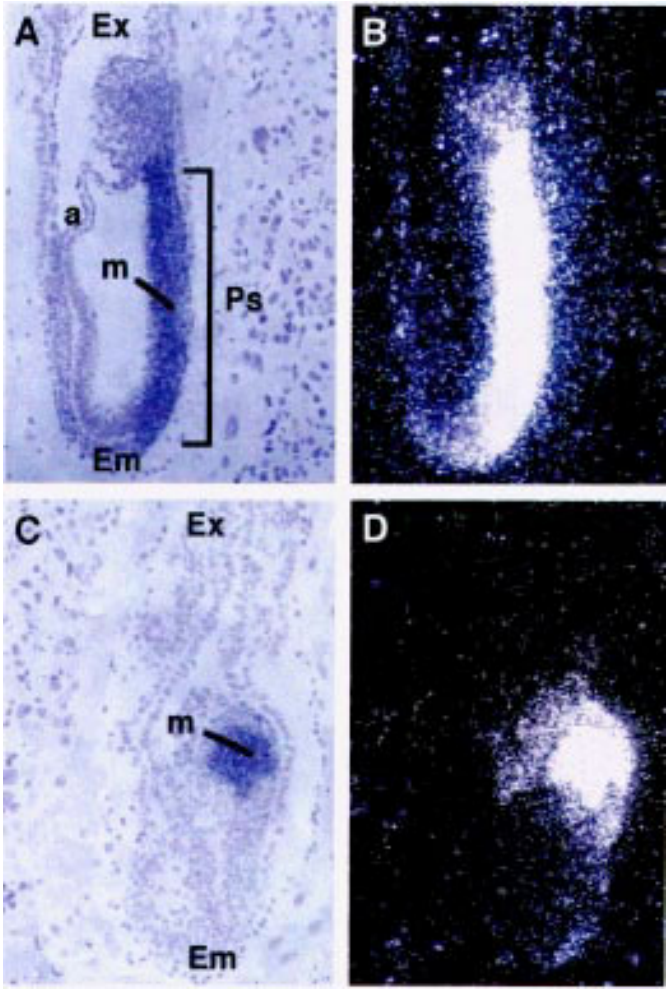


Fig. 3. Gastrulation arrests in HNF-4α –/– mouse embryos. In situ hybridization showing expression of brachyury (T) mRNA in HNF-4α +/+ (*A*, *B*) and HNF-4α –/– (*C*, *D*) mouse embryos at 7.5 days gestation. *A*, *C* Bright field images of hematoxylin and eosin-stained sections show that HNF-4α +/+ mouse embryos generate an amnion (a) and primitive streak (Ps) which are indicative of gastrulation (*A*). However, none of these characteristic morphological features are evident in HNF-4α –/– embryos (*C*). Brachyury mRNA is a marker of nascent mesoderm (m) produced during gastrulation. Dark field images show brachyury mRNA throughout the primitive streak of HNF-4α +/+ embryos (*C*). In HNF-4α –/– embryos (*D*) brachyury +ve cells are limited to a small region at the presumptive posterior end of the embryo. Adapted from Chen et al. [30].

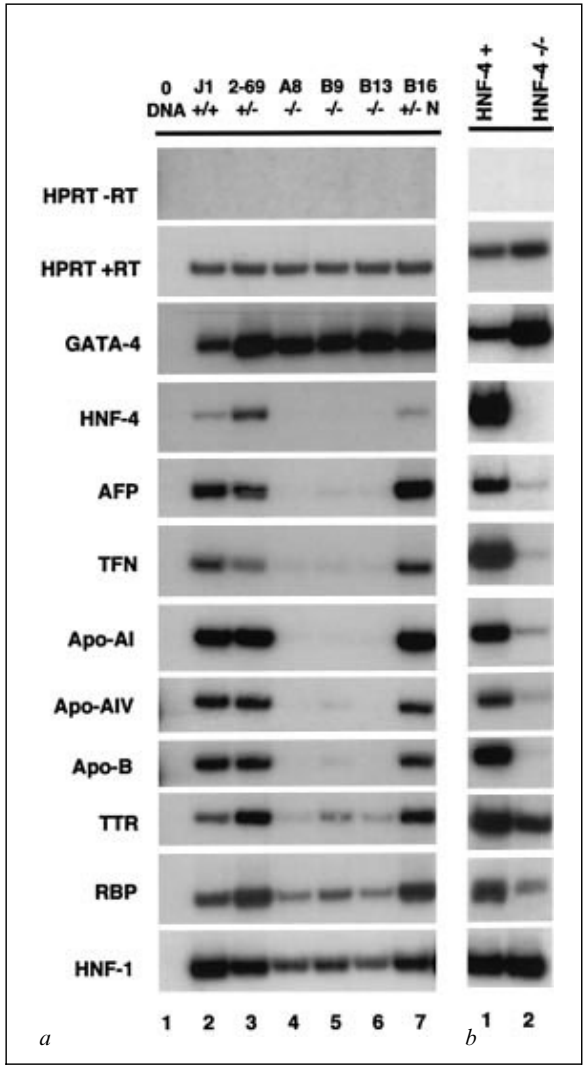


Fig. 4. HNF-4α is a visceral endoderm differentiation factor. Analysis of steady-state mRNA levels encoded by genes expressed in visceral endoderm. *a* RNA from HNF-4α +/+ (J1; lane 2), HNF-4α +/– (2-69, B16; lanes 3 and 7) and HNF-4α –/– (A8, B9, B13; lanes 4, 5 and 6) ES cell embryoid bodies was subjected to RT-PCR [25]. No product was detected using HPRT primers in the absence of reverse transcriptase (-RT) or cDNA (0 DNA; lane 1). Each sample contained equivalent amounts of template since HPRT primers amplified similar levels of product in the presence of RT (HPRT +RT). GATA-4 mRNA levels were similar in all samples showing that each embryoid body preparation contained equivalent amounts of visceral endoderm. Primers to α-fetoprotein (AFP), transferrin (TFN), apolipoproteins-AI (apo-AI), -AIV (apo-AIV), -B (apo-B), transthyretin (TTR), retinol binding protein

of HNF-4α in visceral endoderm differentiation and function, HNF-4α –/– ES cell lines were produced by growing HNF-4α +/– ES cells in high concentrations of G418 [25, 32].

Because HNF-4α is expressed at the onset of visceral endoderm development it seemed possible that HNF-4α could regulate specification of the visceral endoderm lineage. If this was true then the visceral endoderm should be absent from HNF-4α –/– ES cell embryoid bodies. This has been shown to be the case for the transcription factors GATA-4 and GATA-6 [33, 34]. However, a comparison of wild-type and HNF-4α –/– embryoid bodies found that both produced a morphologically distinct endoderm which stained with the diagnostic lectin *Sophora japonicus* agglutinin [25]. This finding was confirmed using RT-PCR to measure steady-state levels of visceral endoderm marker mRNAs: GATA-4 (fig. 4a), HNF-1β and apolipoprotein E [25]. This assay demonstrated that all embryoid bodies, regardless of the presence or absence of HNF-4α, expressed similar levels of these transcripts. Cumulatively, these data demonstrated that HNF-4α is not essential for visceral endoderm specification [25].

Differentiation of the visceral endoderm is sequential process resulting in the expression of characteristic marker genes required for normal visceral endoderm function [35, 36]. To determine whether HNF-4α regulated differentiation of the visceral endoderm, steady-state levels of mRNAs expressed from such genes were measured by RT-PCR. Figure 4 shows that wild-type and HNF-4α +/– embryoid bodies expressed α-fetoprotein (AFP), transferrin (TFN), apolipoprotein-AI (apo-AI), apo-AIV, apo-B, transthyretin (TTR), and retinol binding protein (RBP) mRNA by day 14 of culture. However, in all HNF-4α –/– lines these mRNAs were grossly reduced or absent, demonstrating that HNF-4α is an essential regulator of a wide range of genes required for visceral endoderm function [25]. To determine if this was also the case in vivo, expression of the same genes was compared between HNF-4α +ve and HNF-4α –/– E8.5 mouse embryos. While all genes examined were expressed in HNF-4α +ve embryos, they were again significantly downregulated in HNF-4α –/– embryos (fig. 4b). From this it can be concluded that HNF-4α regulates expression of a large array of genes which define visceral endoderm function. In doing so HNF-4α acts as a visceral endoderm differentiation factor.

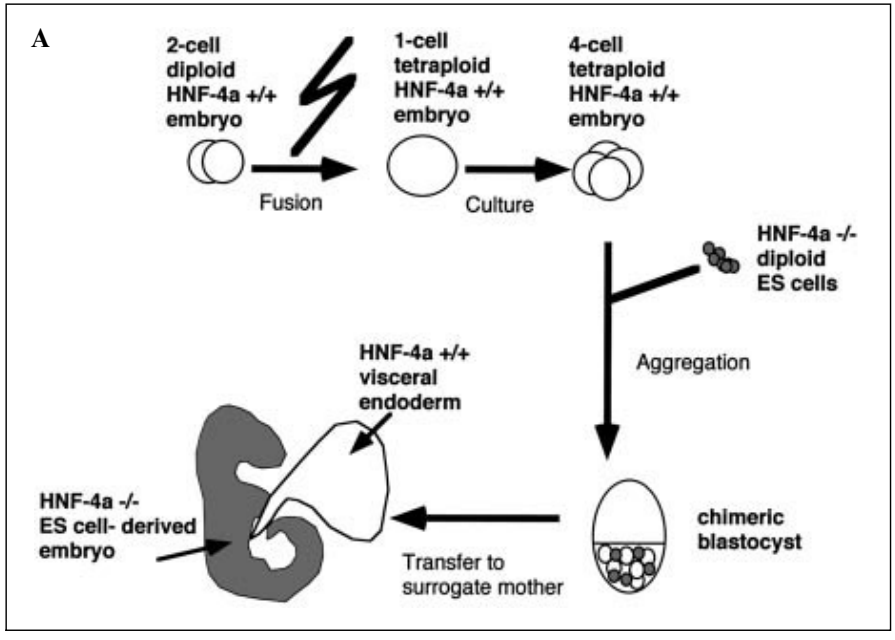
(RBP) and HNF-1α (HNF-1) amplified product in HNF-4α +/+ and +/– embryoid bodies. The level of PCR product amplified by the same primers in HNF-4α –/– embryoid bodies was greatly decreased or undetectable. *b* The same primers were used to detect steady-state mRNA levels in HNF-4α +ve (lane 1) or HNF-4α –/– embryos (lane 2). Adapted from Duncan et al. [25].

Complementation of Defective Visceral Endoderm with HNF-4α +/+ Tissue Rescues the Gastrulation Arrest in HNF-4α –/– Embryos

The demonstration that visceral endoderm lacking HNF-4α expressed greatly reduced levels of secreted serum factors was consistent with the proposal that, in the absence of HNF-4α, disruption of visceral endoderm paracrine activity could block normal gastrulation. If this model were correct specific complementation of HNF-4α –/– embryos with wild-type visceral endoderm should allow HNF-4α –/– embryos to complete the gastrulation process. To achieve this, HNF-4α –/– embryos were generated from HNF-4α –/– ES cells by forming chimeras with tetraploid HNF-4α +/+ embryos [25].

Tetraploid embryos, produced by the fusion of 2-cell stage (diploid) CD-1 embryos, are able to implant but fail to undergo normal development when transplanted into recipient psuedopregnant mothers. However, as illustrated in figure 5a, normal embryogenesis will occur if the tetraploid embryos are aggregated with diploid ES cells prior to implantation. Analysis of the resulting chimeric embryos has shown that the tetraploid cells contribute exclusively to extraembryonic structures, i.e. visceral endoderm, placenta, chorion and trophectoderm lineages, while the embryo proper derives entirely from the diploid ES cells. Postgastrulation ES cell-derived embryos were recovered after 9.5 days of gestation and their genotype ascertained by PCR. Figure 5b shows that these HNF-4α –/– ES cell-derived embryos had completed gastrulation and were almost indistinguishable from wild-type embryos, displaying such distinct postgastrula features as anterior-posterior and dorsal-ventral axes, segmental patterning (somites), neural tube formation, and onset of organo-

Fig. 5. HNF-4α –/– embryos complete gastrulation when complemented with a wild-type visceral endoderm. *A* Diagram illustrating the tetraploid aggregation procedure [48, 49]. Two cell stage HNF-4α +/+ diploid embryos are electrofused to form a single cell tetraploid embryo and are subsequently cultured overnight to form four cell stage embryos. These four cell stage embryos are aggregated with HNF-4α –/– diploid, totipotent embryonic stem cells to produce a chimeric blastocyst. Blastocysts are finally transferred to the uterus of a psuedopregnant surrogate mother where they will continue to develop. The resulting fetuses are derived entirely from the diploid HNF-4α –/– ES cells while the visceral endoderm comes from the HNF-4α +/+ tetraploid embryos [25]. *B–E* Wild-type embryos at 9.5 days gestation (*B*) show distinctive morphological features indicative of a midgestation stage embryo while HNF-4α –/– embryos arrest during gastrulation (*C*) [30]. *D* and *E* HNF-4α –/– ES cell-derived embryos complemented with an HNF-4α +/+ visceral endoderm are virtually indistinguishable from wild-type embryos (*B*). ex = Extraembryonic; e = embryonic; r = rostral; c = caudal; h = heart; l = limb bud; s = somites; n = neural tube. Figure adapted from Duncan et al. [25].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embryo
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diploid
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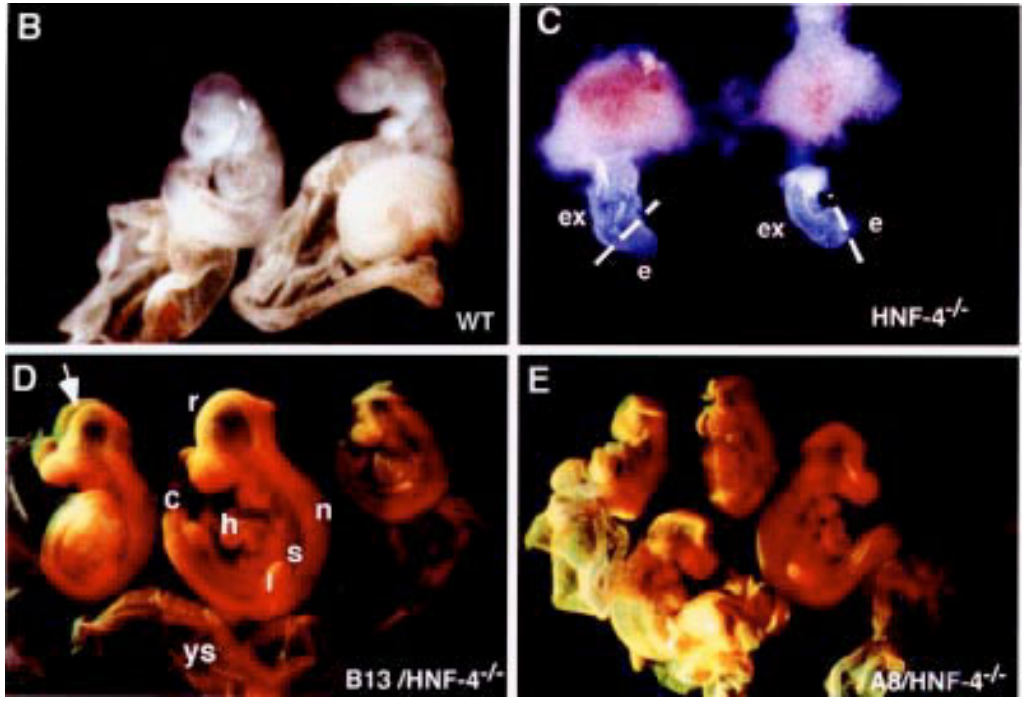
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genesis. In contrast, HNF-4α –/– embryos resulting from a cross of HNF-4α +/– mice appeared grossly abnormal with no morphological signs of gastrulation evident [25, 30].

These data, therefore, establish that HNF-4α –/– cells are competent to generate postgastrulation embryos and that gastrulation requires an HNF-4α +ve visceral endoderm. Since many of the genes whose expression is downregulated in the absence of HNF-4α are serum factors, we propose that, during early stages of postimplantation development in the mouse, a paracrine activity of the visceral endoderm is critical for defining and maintaining an embryonic environment that will support gastrulation.

HNF-4α –/– ES Cell Embryoid Bodies Provide a Model to Identify HNF-4α Target Genes with Roles in Glucose Transport and Metabolism

A nonsense mutation (Q268X) in the HNF-4α gene in humans results in the autosomal dominant form of early-onset NIDDM (MODY1) [37]. To understand how this mutation could result in diabetes a series of biochemical and functional analyses of the HNF-4αQ268X MODY allele were conducted [38, 39]. These studies showed that HNF-4αQ268X protein was transcriptionally inactive and failed to bind DNA. Furthermore, HNF-4αQ268X could not inhibit the transactivation of wild-type HNF-4α even when present in 8-fold excess [38, 39]. From these studies it was concluded that the phenotype in afflicted individuals resulted from a reduction in active HNF-4α levels due to a loss of HNF-4α function in the MODY1 allele.

Because HNF-4α is a transcription factor its likely that haploinsufficiency results in diabetes due to a dysregulation of target gene expression. In order to understand the molecular mechanisms causing the diabetic pathology associated with MODY1 it is, therefore, necessary to identify genes requiring HNF-4α for expression. Ideally such analyses would compare gene expression patterns in HNF-4α +/+ and HNF-4α –/– pancreatic islets. Unfortunately, because HNF-4α –/– embros die during embryogenesis such tissue is currently not available. However, this problem may be circumvented by using the visceral endoderm as an alternative tissue to identify genes which are regulated by HNF-4α [38].

Analyses of gene expression in the visceral endoderm revealed remarkable similarities with that of the liver and pancreatic β-cells [8, 25, 27, 40–42]. Genes expressed in all three cell types included several encoding glycolytic enzymes, metabolic enzymes, glucose transporters, and secreted serum factors [38]. This overlap in gene expression between the visceral endoderm, liver and pancreatic β-cells is likely to reflect the fact that all three contain common

tissue-enriched transcription factors. Amongst these factors are HNF-1α and -1β, HNF-3α, -3β and -3γ, and HNF-4α [27, 28, 41, 43, 44]. Furthermore, the visceral endoderm is known to secrete insulin and expression of several genes is responsive to serum glucose levels [38, 45–47]. This suggests that molecular pathways which are critical for the glucose sensing function of the pancreatic β-cells are intact in the visceral endoderm. Therefore, because the visceral endoderm has such extensive molecular and functional overlap with the liver and pancreas it provides an accessible, genetically manipulable model to identify HNF-4α target genes whose action may be pertinent to the regulation of glucose metabolism [38].

The mRNA levels of 60 genes known to be linked to insulin secretion and/or glucose metabolism were measured in HNF-4α +/+, HNF-4α +/– or HNF-4α –/– ES cell embryoid bodies. Strikingly, the expression of four genes involved in glucose homeostasis was either downregulated or absent in HNF-4α –/– visceral endoderm compared to HNF-4α +/+ visceral endoderm [38]. Moreover, the same result was obtained from a comparison of HNF-4α +ve to HNF-4α –/– embryos at 8.5 days gestation. Particularly significant was the finding that expression of the glucose transporter GLUT2 and the glycolytic enzyme aldolase b genes was grossly downregulated in the absence of HNF-4α. These data demonstrate that HNF-4α is an important regulator of genes involved in both glucose sensing and glycolysis and offers a mechanism whereby loss of HNF-4α activity could disrupt glucose homeostasis [38].

Discussion

The use of genetics has provided an invaluable tool for the investigation of gene function, not only in the mouse but in species ranging from bacteriophages through humans. Analysis of mutants generally uncovers the first essential role of a gene and later functions are often masked by earlier lethal phenotypes. In the case of HNF-4α, its first role during development is critical for normal gastrulation, a process which is essential for subsequent organogenesis. HNF-4α –/– emmbryos will proceed through gastrulation, however, if they are complemented with a wild-type (HNF-4α +/+) visceral endoderm. This can be achieved by aggregation of tetraploid embryos with HNF-4α –/– ES cells. Using this approach it should now be possible to produce midgestation stage HNF-4α –/– embryos and use them to answer whether HNF-4α is required for liver development.

Apart from glucokinase all the MODY genes described to date are transcription factors and all of those transcription factors are expressed in the visceral endoderm in addition to pancreatic islets and liver. It is likely that

the diabetes associated with those MODY patients having mutations in transcription factor genes is due to a pleiotropic dysregulation of target gene expression. Some of these target genes are likely to have direct roles in regulating glucose metabolism, such as aldolase b, and mutations in them may contribute to the more prevalent noninsulin-dependent forms of diabetes. To identify the targets regulated by the MODY transcription factors using traditional molecular approaches is difficult due to the lack of availability of pancreatic islet tissue. However, the similarity of gene expression patterns in the visceral endoderm, liver and pancreatic β-cells may provide an alternative approach. Visceral endoderm can be obtained from ES cell embryoid bodies harboring mutations in specific MODY transcription factors and this can be used to identify target genes. As shown above this was used successfully to identify aldolase b and GLUT2 as genes requiring the MODY1 transcription factor HNF-4α for their expression. Using this approach it is likely that many new targets of MODY transcription factors will be identified and some of these will have important roles in regulating glucose homeostasis.

Acknowledgments

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Stephen A. Duncan, Department of Cell Biology, Neurobiology and Anatomy,
Medical College of Wisconsin, 8701 Watertown Plank Rd, Milwaukee, WI 53226 (USA)

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HNF4 as a Liver Gene Transcription Factor

John M. Stafford, Jen-Chywan Wang, Donald K. Scott, Takashi Sugiyama, Daryl K. Granner

Department of Molecular Physiology and Biophysics, Vanderbilt University Medical School, Nashville, Tenn., USA

Hepatocyte nuclear factor 4α (HNF4α), a member of the nuclear receptor superfamily of transcription factors, is required for the tissue-specific expression of a variety of metabolic genes in the liver, kidney, and pancreatic β-cells [1]. Various inherited mutations of the HFN4α gene lead to impaired insulin secretion and autosomal dominant forms of type 2 diabetes mellitus, specifically maturity onset diabetes of the young (MODY) type 1 [2]. In addition to its causal role in MODY-1, HNF4α is involved in a regulatory hierarchy involving the other known MODY gene products, such as HNF1, and thus may contribute to the pathogenesis of diabetes on several levels [3–5].

HNF4α regulates the expression of many genes that control glucose homeostasis, including liver-type pyruvate kinase, GLUT2, GAPDH, and phosphoenolpyruvate carboxykinase (PEPCK) [6, 7]. Glucose-tracer studies in rodents and humans have shown that unrestrained gluconeogenesis is a major source of the increased glucose production in noninsulin-dependent diabetes mellitus (NIDDM) [8, 9]. PEPCK catalyzes the conversion of oxaloacetate to phosphoenolpyruvate, a rate controlling step in gluconeogenesis [10, 11]. PEPCK mRNA, protein abundance and activity are increased in animal models of NIDDM and streptozotocin-induced diabetes [12, 13]. Furthermore, overexpression of PEPCK in transgenic animals results in hyperglycemia and a NIDDM-like condition [14, 15]. Together, these observations indicate that tight regulation of PEPCK is necessary to maintain euglycemia. The regulation of PEPCK occurs primarily at the level of transcription. Basal PEPCK gene transcription is regulated in part by HNF1, and glucocorticoid induction is regulated in part by both HNF4 and HNF3 [7, 16–18]. Furthermore, Hanson

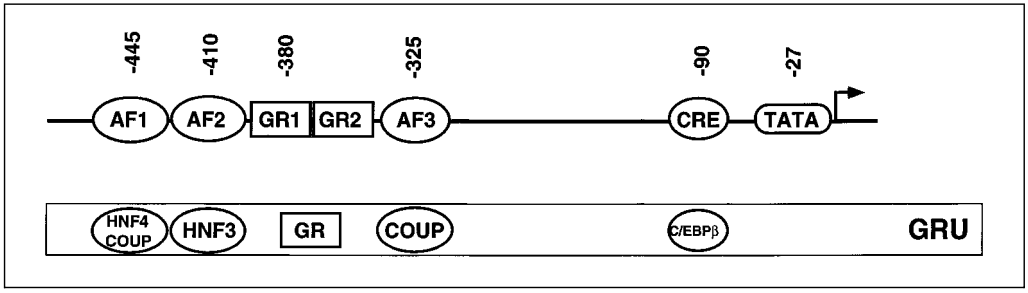

Fig. 1. Schematic diagram of the PEPCK GRU. The PEPCK GRU includes two GR-binding sites (GR1 and GR2) and four accessory elements (AF1, AF2, AF3 and the CRE), and their associated transacting factors (lower panel). The position of each *cis*-element, relative to the transcription initiation site, is indicated above the figure.

and colleagues suggest that glucorticoids play a dominant role in determining the extent of hepatic PEPCK gene transcription in diabetes [19]. Because of the role that hepatic nuclear factors (HNFs) have in the transcriptional regulation of the PEPCK gene, analysis of their role in this regulation provides a good system for studying how MODY gene products interact to control gluconeogenesis, and thus glucose homeostasis.

Hepatic PEPCK is under tight metabolic control. Glucocorticoids, glucagon (through cAMP), and retinoic acid increase the rate of PEPCK gene transcription while insulin and glucose inhibit transcription [10, 11, 20]. Induction of PEPCK gene transcription by glucocorticoids (the glucocorticoid response) is achieved by a glucocorticoid response unit (GRU), comprised of two glucocorticoid receptor binding sites (GR1 and GR2), and binding sites for at least four other sets of transciption factors, termed accessory factors (these sites are designated AF1, AF2, AF3 and the CRE) (fig. 1). The GR binding elements, GR1 and GR2, alone or in combination are unable to confer glucocorticoid responsiveness when placed in front of a heterologous promoter [21]. Instead, a complete glucocorticoid response requires a full set of accessory factors (AFs) binding their cognate DNA elements (fig. 1). Mutation of any one AF binding element in the PEPCK promoter causes a 50–70% reduction in the glucocorticoid response. Any combination of two mutations of AF1/AF3, AF2 or the CRE essentially abolishes the glucocorticoid response [21, 22]. Thus, PEPCK GR1 and GR2, unlike simple glucocorticoid response elements (GREs), are inactive in the absence of at least two accessory elements. The proteins that bind the AF elements and mediate AF activity to the glucocorticoid response have been identified. HNF4 and chicken ovalbumin upstream promoter transcription factor (COUP-TF) bind the AF1 element [7]. HNF3

family members bind the AF2 element [18]. COUP-TF binds the AF3 element, and CEBP-β binds the CRE [23; Yamada, et al., J Biol Chem, in press]. Furthermore, the AF1/AF3 and AF2 elements cannot replace each other or be switched in position relative to the GREs [22]. Taken together, these findings show that each AF is specifically required and that a precise organization of these factors in the GRU is necessary for a full glucocorticoid response.

Results

The distinct and specific role of the AF1 element in the GRU prompted us to localize the domains required for the accessory factor activity of the AF1 binding factor, HNF4. This analysis requires that the function, or lack thereof, of various mutations of the HNF4 molecule be studied in the cells of interest. H4IIE rat hepatoma cells have large amounts of endogenous HNF4, thus it is difficult to analyze the effect of transiently transfected HNF4 mutants in the presence of the wild-type protein. However, all mammalian cells lack the yeast transcription factor GAL4. Thus an expression plasmid encoding the GAL4 DBD ligated to various portions of HNF4 can be used to specifically tether these HNF4 mutants to a reporter gene containing a GAL4 DNA-binding element. To this end, a reporter gene construct was made wherein the AF1 element of the PEPCK promoter was replaced by a yeast GAL4-binding site (pGAL4-AF1) in the context of an otherwise wild-type promoter (–467 to +69). Wild-type HNF4 cannot bind to this element, thus effectively removing it from the analytical system. Glucocorticoid-responsive H4IIE cells were transfected with this reporter and the glucocorticoid response obtained was about 30% that of transfected wild-type PEPCK-CAT reporter, pPL32 (fig. 2A) [24]. This reduction is of similar magnitude to that observed when the AF1 element is deleted or mutated [23].

HNF4 has two transactivation domains, TAD1 (a.a. 1–24) and TAD2 (a.a. 128–374) [25] (see footnote). A variety of expression constructs were made wherein various portions of HNF4 were ligated to the GAL4-DNA-binding domain (DBD), to test which domains of HNF4 are necessary to restore the glucocorticoid response to the pGAL4-AF1 reporter construct. Cotransfection of H4IIE cells with the pGAL4-AF1 reporter and a construct that encodes the GAL4-DBD alone gave no increase in the glucocorticoid response mediated by this reporter, however a plasmid that contains full-length HNF4 ligated to the GAL4-DBD restored the glucocorticoid response (fig. 2B, lines 1 and 2). GAL4-HNF4 (1–45) was not able to restore the glucocorticoid response (fig. 2B, line 3). GAL4-HNF4 (1–374) and GAL4-HNF4 (128–374) were able to mediate AF1 activity, and thus restore the glucocorticoid response

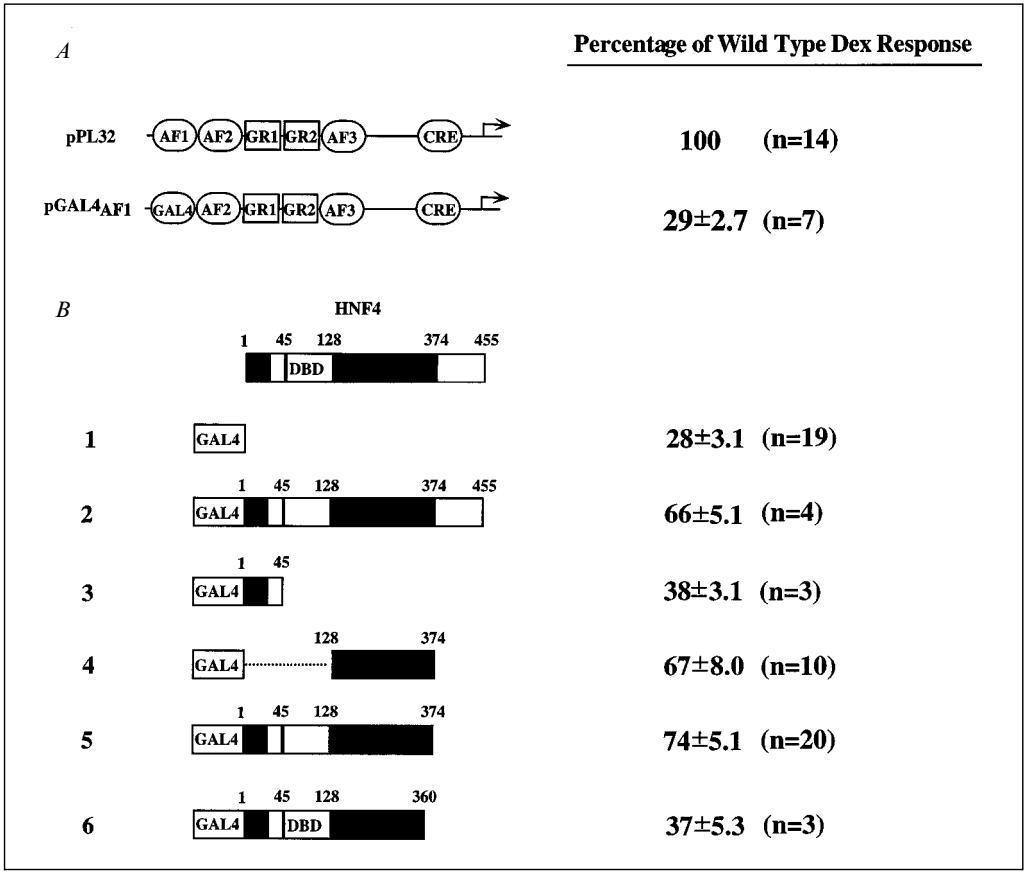

Fig. 2. The C-terminal transactivation domain of HNF4 is sufficient for AF1 activity. *A* H4IIE cells were transfected with pPL32, the wild-type PEPCK promoter reporter construct, or pGAL4AF1, wherein the AF1 element is replaced with a GAL4-binding site. All cells were cotransfected with the GR expression vector and were treated with or without 500 n*M* dexamethasone (DEX) for 18–24 hours. CAT activity was then measured as described above. Results are presented relative to the wild-type glucocorticoid response (pPL32; top panel) and represent the average ± SE of the number of experiments indicated. *B* An expression plasmid encoding one of the various GAL4-HNF4 fusion proteins, and the GR expression vector, were cotransfected with $pGAL4_{AF1}$. A schematic representation of the structure of the HNF4 protein is shown. The shaded areas represent the transactivation domains of HNF4.

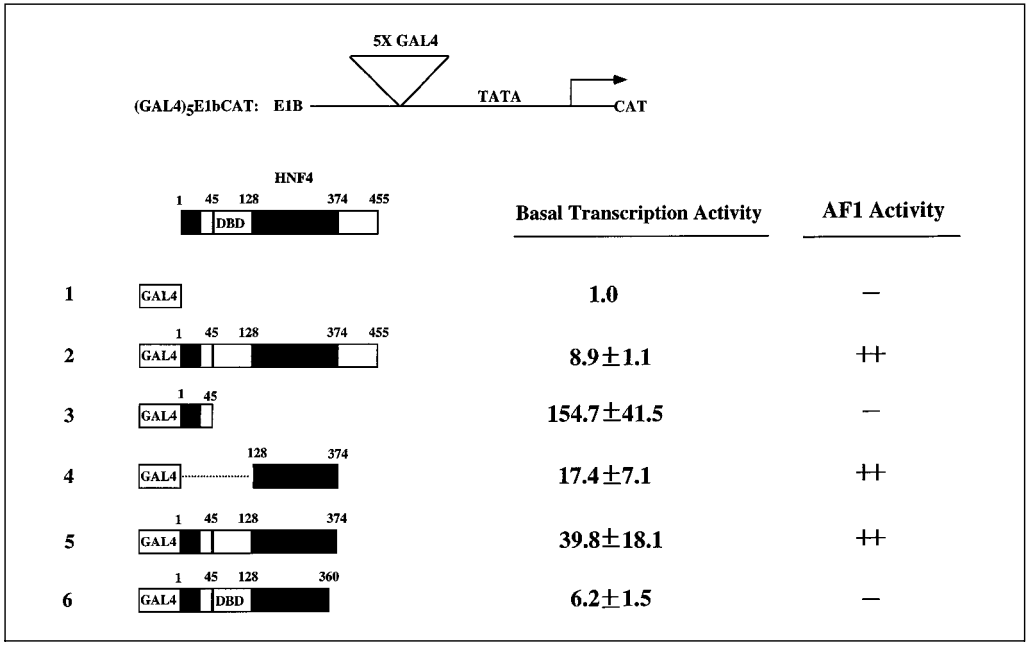

Fig. 3. Comparison of the basal transactivation and AF1 activities of various GAL4-HNF4 fusion proteins. Expression plasmids (2 μg) that encode various GAL4-HNF4 fusion constructs were cotransfected into H4IIE cells with a reporter construct (5 μg) that contains five tandem copies of the GAL4-binding site inserted into the E1b promoter [$(GAL4)_5$E1bCAT]. Results are presented relative to the activity of GAL4 DBD protein and represent the average ± SE of at least five experiments. The AF1 activity of each GAL4-HNF4 fusion protein is also shown.

(fig. 2B, lines 4 and 5). However, it appears that the full TAD2 domain is required for AF1 activity, as GAL4-HNF4 (1–360) which contains only TAD1 did not restore the glucocorticoid response over the DBD alone (fig. 2B, line 6).

Next, the GAL4-HNF4 fusion proteins described in the previous experiments were cotransfected with the $(GAL4)_5$-E1b-CAT reporter construct into H4IIE cells. This reporter construct contains five copies of the GAL4-binding site fused to the minimal E1b promoter, and was used to determine whether there is a correlation between the ability of HNF4 transactivation domains to confer PEPCK accessory factor activity with basal transactivation of a heterologous promoter. The transactivation potential of GAL4-HNF4 (1–374), (128–374), and (1–360) correlates with the ability of these regions to provide PEPCK AF1 activity (fig. 3, lines 4–6) [24]. GAL4-HNF4 (1–45),

which was the strongest transactivator of the $(GAL4)_5$-E1b-CAT reporter, demonstrated very low PEPCK AF1 activity (fig. 3, line 3). In contrast, GAL4-HNF4 (128–374) mediates PEPCK AF1 activity but its transactivation activity is about 8-fold lower than that of GAL4-HNF4 (1–45) (fig. 3, lines 3 and 4).

It is not known why the TAD2 domain of HNF4 can confer AF1 activity but not transactivate efficiently through the $(GAL4)_5$-E1b-CAT reporter. It is possible that this selectivity arises because TAD2 must recruit other coregulatory molecules that are required for the PEPCK glucocorticoid response. Interestingly, this accessory function mediated by GAL4-HNF4 (128–374) can be replaced by chimeric proteins consisting of the GAL4 DBD and the activation domains of E1A or VP16 [24], suggesting a protein-protein interaction in which these diverse activation domains recruit a common, or at least functionally redundant, coregulatory molecule. Thus, the PEPCK gene GRU consists of a number of specific protein-DNA and protein-protein interactions involving the GR, AFs, and possibly coactivator molecules.

Several coactivators, including CBP/p300, and members of the p160 family such as SRC-1 and GRIP1/TIF2 interact with the TAD2 domains of nuclear receptors and augment their transactivation potential [26]. These molecules seemed like possible candidates for coactivation with HNF4. We used the yeast two-hybrid assay system to determine if SRC1 and GRIP-1 interact with HNF4 in vivo, and thus might serve as intermediary molecules in conferring accessory factor activity to the PEPCK promoter. The yeast-two hybrid assay uses a β-galactosidase reporter gene positioned downstream from a GAL4-binding site. The yeast transcription factor GAL4, which binds to the GAL4 site and transactivates this reporter gene, has both a DNA-binding domain (DBD) and an activation domain (AD). This assay works because transcription factors are functionally modular and the DBD and AD can be separated, then by subcloning, ligated to the domains of the proteins to be tested for interaction. Thus, in order to activate expression of this reporter, the 'bait' molecule ligated to the GAL4-DBD must interact with the 'prey' molecule fused to the GAL4-AD. We used the GAL4-DBD fused to HNF4 as 'bait', and the GAL4-AD fused to SRC1 or GRIP-1 as 'prey' to test if HNF4 interacts with these coactivators in vivo. The expression of GAL4-HNF4 (128–374) or the GAL4-HNF4 (1–360) in yeast cells failed to activate the β-galactosidase reporter gene (data not shown). Thus, neither of these chimeric proteins possesses intrinsic transcriptional activity in yeast. Further, β-galactosidase activity was not observed when an expression plasmid (pGAD-424) that encodes the GAL4-AD was coexpressed with either of these two GAL4-HNF4 fusion proteins, which indicates that the GAL4-AD itself has no affinity for the HNF4 fusion proteins (data not shown). However, reporter gene activity was strongly induced by coexpression of a chimeric protein which contains

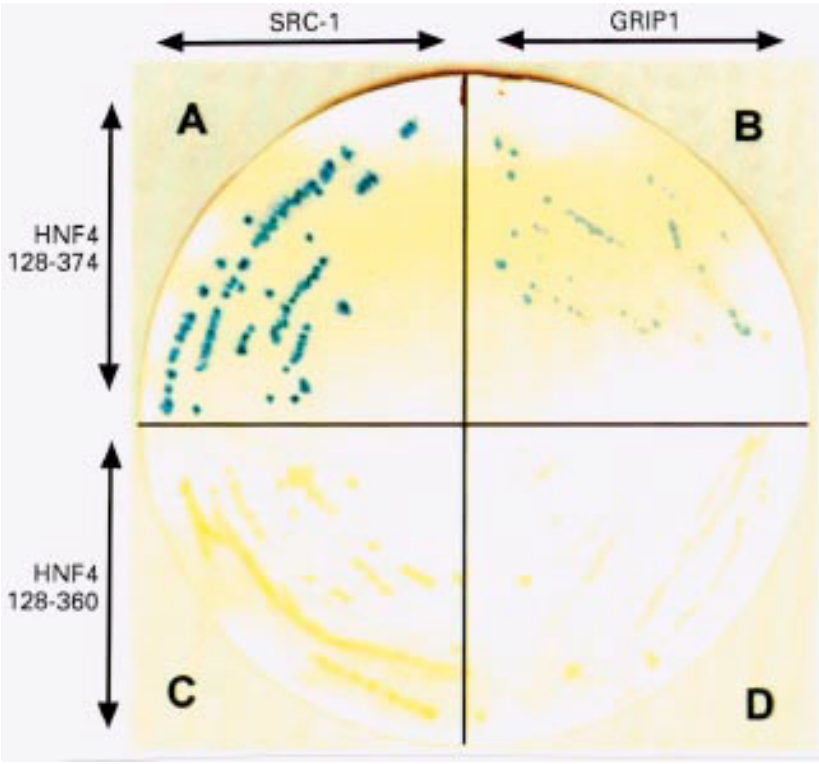

Fig. 4. In vivo protein-protein interactions between GAL4-HNF4 chimeric proteins and SRC-1 or GRIP1. Yeast cells were cotransformed with combinations of the yeast plasmids, pGBT9-HNF4 (128–374), pGBT9-HNF4 (128–360), pGAD424 GRIP1/FL or pGAD424 SRC-1, as indicated, and were grown on synthetic dropout plates lacking Leu and Trp. Yeast colonies were then subjected to a colony-filter assay for β-galactosidase activity. Yeast cells that contained pGBT9-HNF4 (128–374) and pGAD424-SRC-1a turned dark blue 30 minutes after the reaction was initiated. Yeast cells that contained pGBT9-HNF4 (128–374) and pGAD424-GRIP1 turned blue 4 hours after the reaction was initiated. In liquid β-galactosidase activity assays, yeast that contain the GAL4 AD SRC-1 fusion protein and GAL4 HNF4 (128–374) showed stronger activity than yeast that contain the GAL4 AD GRIP1 fusion protein and GAL4 HNF4 (128–374) (data not shown).

the GAL4-AD fused to either SRC-1 or GRIP1 and GAL4 HNF4 (1–374) (fig. 4A, B) [27]. Coexpression of either the SRC-1 or GRIP1 fusion proteins with GAL4-HNF4 (1–360) did not induce β-galactosidase activity (fig. 4C, D). These results suggest that both SRC-1 and GRIP1 interact with HNF4 and that these interactions are dependent on the presence of a critical region of the TAD2 domain (a.a. 360–374) of HNF4.

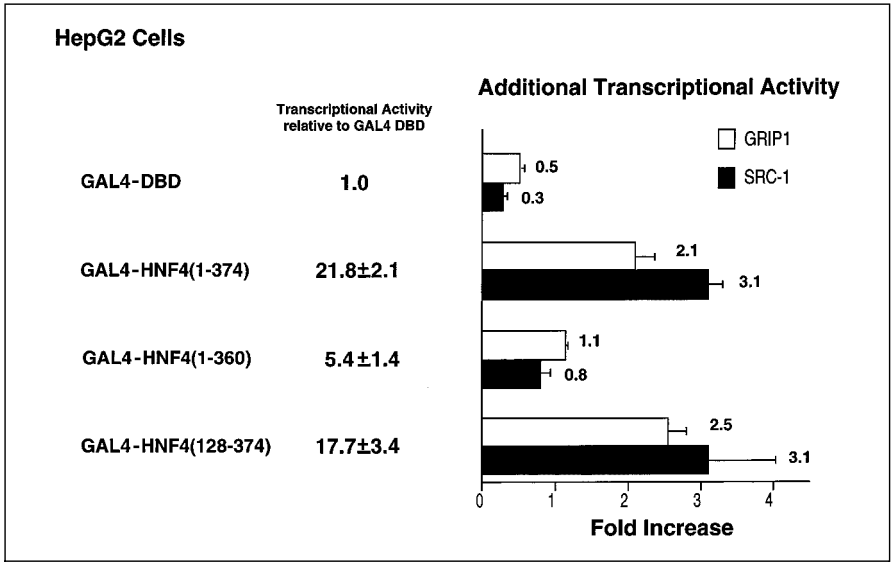

Fig. 5. SRC-1 and GRIP1 potentiate the activity of GAL4-HNF4 fusion proteins. Expression plasmids that encode SRC-1 or GRIP1 (5 μg) and GAL4-HNF4 fusion proteins (0.5 μg), were cotransfected into HepG2 cells along with the reporter construct $(GAL4)_5$E1bLuc (2.5 μg). The transcriptional activity of each GAL4-HNF4 fusion protein is expressed as the fold increase relative to the activity of the GAL4-DBD. The additional increase of activity provided by SRC-1 or GRIP1 is presented relative to the activity of the GAL4-HNF4 fusion proteins obtained when equal amounts of DNA from the control vectors were transfected. These results represent the mean ± SEM of at least three experiments.

Expression plasmids that encode either SRC-1 or GRIP1 were then cotransfected into HepG2 hepatoma cells with plasmids that encode the GAL4-HNF4 fusion proteins previously described, and with the reporter gene $(GAL4)_5$-E1b-LUC to determine if there is a functional coactivation of HNF4 by these molecules. As in the previously described experiments, the activity of each GAL4 chimeric protein was measured relative to the GAL4-DBD. Overexpression of SRC-1 or GRIP1 did not augment the transactivation potential provided by the GAL4 DBD in this system (fig. 5) [27]. As noted previously, both GAL4-HNF4 (1–374) and (128–374) possess a strong transactivation potential in the context of the $(GAL4)_5$-E1b promoter and confer AF1 activity to PEPCK, whereas GAL4-HNF4 (1–360) is a weak transactivator of $(GAL4)_5$-E1b and does not confer PEPCK AF1 activity. Overexpression of SRC-1 potentiated the transactivation potential of GAL4-HNF4 (1–374) an additional 3-fold. SRC1 also augmented GAL4-HNF4

(128–374), which contains only the TAD2 domain, about 3-fold (fig. 5). In contrast, GAL4-HNF4 (1–360), which contains TAD1 but lacks the critical portion of TAD2 (360–374), was not influenced by the overexpression of SRC-1 (fig. 5). Overexpression of GRIP1 also increased the transcriptional activity of GAL4-HNF4 (1–374) and (128–374), but not GAL4-HNF4 (1–360) (fig. 5).

We next tested the ability of SRC-1 and GRIP1 to potentiate the transactivation potential of these three GAL4-HNF4 fusion proteins in HeLa cells in order to determine if this coactivation of HNF4 by SRC-1 and GRIP 1 is cell-type specific. SRC-1 and GRIP1 both increased the transactivation potential of GAL4-HNF4 (1–374) and (128–374), but not (1–360) (data not shown) [27]. Thus, coactivation of these GAL4-HNF4 fusion proteins by SRC-1 and GRIP1 is not stringently dependent on the cellular environment. These results show that both SRC-1 and GRIP1 can act as coactivators for HNF4 and that these effects are dependent on a.a. 360–374 of TAD2 in HNF4. These results are consonant with the dependence on the same region of TAD2 for in vivo binding of these coactivators with HNF4 seen in the yeast two-hybrid assay (fig. 4).

Another coactivator, CREB Binding Protein (CBP) associates with HNF4 and potentiates the activity of a reporter construct that contains multiple HNF4 binding sites [28]. We tested if p300, a protein closely related to CBP, potentiates the activity of GAL4-HNF4 chimeric proteins. Overexpression of p300 in HeLa cells enhanced the activity of GAL4-HNF4 (128–374) but not GAL4-HNF4 (1–360). Thus, like SRC-1 and GRIP-1, coactivation of HNF4 by p300 requires TAD2. The transactivation potential of GAL4-HNF4 (128–374) was synergistically enhanced when both SRC-1 and p300 were overexpressed (fig. 6) [27]. A similar synergistic activation by SRC-1 and p300 has been observed with other nuclear receptors [29, 30].

A regulatory hierarchy of hepatocyte nuclear factors has been described wherein HNF4 binds to its cognate DNA element in the HNF1 promoter and enhances HNF1 gene transcription [3, 4]. If the HNF4-coactivator interactions described in the previous figures are functionally important, the coexpression of either SRC-1 or GRIP1 should enhance HNF1 promoter activity. In fact, overexpression of SRC-1 in HepG2 hepatoma cells increased HNF1 gene promoter activity nearly 4-fold (fig. 7). As seen in the experiments shown in figure 4, GRIP1 was also slightly less effective in activation of the HNF1 promoter (fig. 7) [27]. Deletion of the HNF4-binding site in the HNF1 promoter (HNF1ΔA) resulted in about a 90% reduction of HNF1-reporter gene expression, as expected [4]. Overexpression of either SRC-1 or GRIP1 was not able to potentiate HNF1 promoter activity in the absence of the HNF4-binding site (fig. 7).

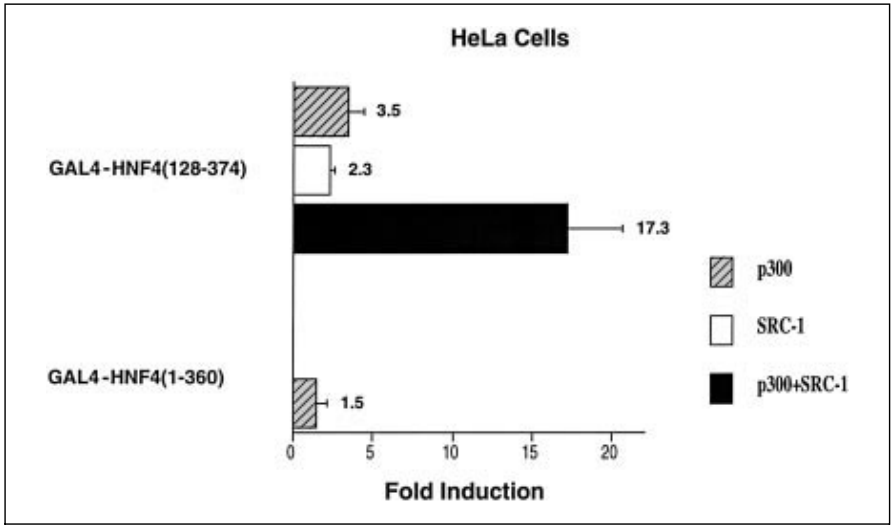

Fig. 6. SRC-1 and p300 act synergistically to increase the activity of GAL4-HNF4 (128–374). Expression plasmids that encode p300 (10 μg), SRC-1 (10 μg), and GAL4-HNF4 fusion proteins (4 μg) were cotransfected into HeLa cells with the $(GAL4)_5$E1bLuc reporter construct (2 μg). The data are expressed as the fold induction relative to the activity of the GAL4-HNF4 fusion proteins in cells in which SRC-1 or GRIP1 was not overexpressed. These results represent the mean ± SEM of at least three experiments.

Discussion

The transcription factor, HNF4, a member of the nuclear receptor superfamily, is important for tissue-specific gene expression, including several genes involved in glucose homeostasis. One example is the involvement of HNF4 in glucocorticoid-mediated induction of PEPCK gene transcription. In this paper, we summarize data showing that AF1 activity is conferred by the TAD2 activation domain of HNF4 (a.a. 128–374). Loss of a critical portion of TAD2 (a.a. 360–374) leads to a loss of basal transactivation of the $(E1b)_5$-GAL4-LUC reporter and loss of accessory factor activity in the PEPCK glucocorticoid response. Further, we show that this region of the TAD2 domain is critical both for functional coactivation of HNF4 by SRC-1 and GRIP1, and also for in vivo binding of HNF4 to SRC-1 and GRIP1. In addition, the data presented shows that p300 acts synergistically with SRC-1 to further enhance the transcriptional activity of HNF4. Recent studies suggest that transactivation by nuclear receptors requires a complex of activator and coactivator molecules, including as many as 30 accessory factors and numerous coacti-

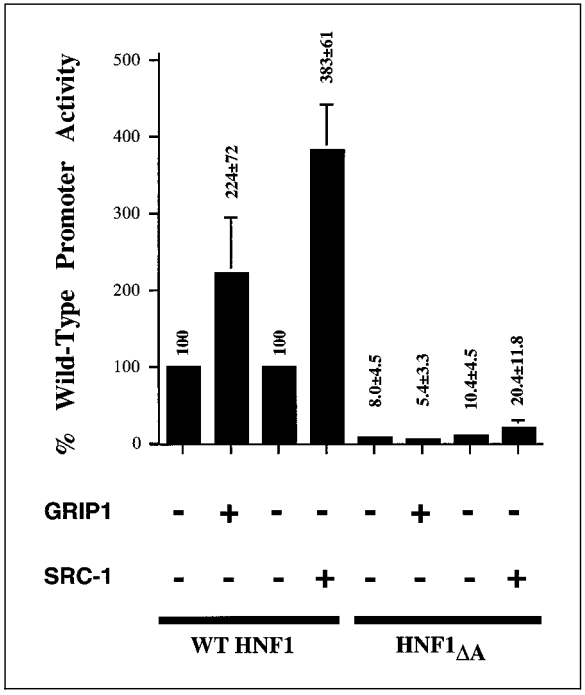

Fig. 7. SRC-1 and GRIP1 potentiate transactivation through the HNF1 gene promoter. Expression plasmids that encode SRC-1 (20 μg) or GRIP1 (5 μg), and a reporter construct that contains either the wild-type HNF1 gene promoter (2.5 μg) or the promoter which lacks a functional HNF4-binding site (ΔA) were cotransfected into HepG2 cells. Results are presented relative to wild-type HNF1 promoter activity when equal amounts of control plasmid were transferred (100%). These results represent the mean ± SEM of at least three experiments.

vators, including SRC-1/GRIP1, p300/CBP, and p/CAF [31–34]. Experiments are underway to test if a similar complex is involved in the PEPCK gene glucocorticoid response.

The transcription factor COUP-TF also serves as an accessory factor in mediating the glucocorticoid response of PEPCK gene transcription by binding both the AF1 and AF3 elements [7, 23]. HNF4 and COUP-TF can both bind the AF1 element, but only COUP-TF binds to the AF3 element to confer accessory factor activity [23]. We have recently shown that COUP-TF also interacts with SRC-1 [T Sugiyama, and DK Granner, submitted]. Our findings suggest that the accessory factor role of HNF4 and COUP-TF in the GRU may be to recruit specific coactivators, such as SRC-1. This may contribute

to the formation of protein-DNA and protein-protein complexes specifically required for the induction of PEPCK gene transcription by glucocorticoids. If this is the case, ligand bound GR may act as a signal transducer that triggers assembly of this nucleoprotein complex rather than solely as a transactivator, since neither GR1 nor GR2 can mediate glucocorticoid induction of the PEPCK gene (or of other tested reporters) by themselves [21].

A simple GRE represents an all-or-none switch, whereas the PEPCK GRU allows for a graded response in which the multiple GR binding sites and accessory factors provide intricate regulation. Additionally, the multiple factors that bind the GRU and contribute to the glucocorticoid response provide a functional overlap to ensure glucocorticoid responsiveness of the promoter. This may be the reason for the seemingly redundant functions of HNF4 and COUP-TF in mediating AF1 activity. Interestingly, the PEPCK GRU is part of a much more complex assembly of elements and factors that we have termed a metabolic control domain (MCD) [35]. The MCD of the PEPCK gene consist of overlapping hormone response units (HRUs) for glucocorticoids, cAMP, retinoic acid, and insulin. These HRUs, in turn, are each comprised of multiple transcription factor binding elements. Some of these elements bind more than one set of proteins and participate in more than one HRU. For example, the AF1 element, which binds HNF4 and COUP-TF as accessory factors for the glucocorticoid response also binds RAR/RXR heterodimers to mediate retinoic acid induction of PEPCK gene transcription [7, 36–38]. Thus, the MCD provides the organism with the means of establishing an integrated response to the demands of tightly controlled gluconeogenesis.

HNF4 regulates the transcription of many genes involved in glucose homeostasis in addition to PEPCK. While the mechanism of HNF4 gene mutations in the pathophysiology of MODY (type 1) has not been established, it is of interest to note that HNF4 is required for expression of HNF1, another MODY gene (type 3) [3, 4, 39]. Inherited heterozygous mutations in either HNF4 or HNF1 lead to impaired insulin secretion in humans [2, 39]. In contrast, mice deficient in just one allele of either HNF4 or HNF1 do not have demonstrable defects in glucose homeostasis [6, 40]. Recently, Polonsky and colleagues have shown that HNF1 knockout mice have defects in glucose flux through glycolysis in pancreatic β-cells, which may lead to the impaired insulin secretion seen in these animals [41]. Although humans may be more sensitive to reductions in gene dosage of HNF1, this finding suggests that a similar defect in glycolysis may account for the impaired insulin secretion detected in MODY3 patients. Interestingly, at least one human MODY pedigree has a mutation of the HNF4-binding site in the HNF1 promoter [42]. These patients presumably have a loss of HNF1 function because of the inability of HNF4 to properly transactivate through this pro-

moter. Thus, a common denominator of MODY types 1 and 3 may be the loss of HNF1 gene function. In this regard, it may be noteworthy that we found that over expression of both SRC-1 and GRIP1 increases the activity of the HNF1 promoter, and that a mutation of the HNF4-binding site in this promoter disrupts this effect.

It has become evident in study of the MODY genes, that there is a complex regulatory network of hepatic and pancreatic transcription factors, including HNF1, HNF3, HNF4 and PDX1 [5, 43]. All of these gene products, in turn, regulate many other genes involved in glucose homeostasis. Several genes involved in glycolysis and gluconeogenesis, those encoding glucose 6 phosphatase, GADPH, aldolase B, liver-pyruvate kinase, and PEPCK, are normally regulated by HNF4 or HNF1 [6, 7, 44]. In the pancreas, the deficiency of HNF1 (and presumably HNF4) principally affects glycolysis. In the liver, mutations in HNF1 or HNF4 may give rise to defects in both glycolysis and gluconeogenesis, thus the MODY phenotype (types 1 and 3) could involve both liver and pancreatic defects.

The effect of mutations in transcription factors that regulate PEPCK (e.g. HNF1, HNF3, or HNF4) might be predicted to decrease PEPCK gene transcription, and thus gluconeogenesis. As noted previously, however, PEPCK enzyme activity, protein, and mRNA are elevated in animal models of type II diabetes [12, 13]. Despite dramatic changes in the levels of other metabolic genes regulated by HNF4 and HNF1 in MODY [6], PEPCK gene transcription may be only slightly affected. HNF4 mediates glucocorticoid induction of PEPCK gene transcription through the AF1 element. As mentioned previously, COUP-TF also binds the AF1 element and appears to be functionally redundant in mediating accessory factor activity. Thus, loss of HNF4 in the liver would not be predicted to have a large effect on PEPCK gene transcription. Further, it is unclear whether the HNFs involved in MODY are limiting for the requirements of PEPCK gene transcription. For instance, even with a 50% reduction in the levels of HNF1 in MODY3, there may still be enough HNF1 for normal regulation of the PEPCK gene. Other metabolic genes without such functional redundancy of regulatory molecules may be more affected. Indeed, changes in the regulation of other metabolic genes due to mutations in these HNFs may have a large, though more indirect, effect on PEPCK gene regulation. For instance the impaired insulin secretion in MODY1 and MODY3 would relieve insulin's repressive effect on PEPCK gene transcription. Thus mutations in MODY may actually give rise to increased PEPCK levels, and thus increased gluconeogenesis and hyperglycemia. Patients with MODY2 (GK) have been shown to have impaired glycogen storage and increased gluconeogenesis [45]. The contribution of gluconeogenesis to the more severe clinical course of MODY types 1 and 3 is unknown.

Footnote

To avoid confusion in terminology with PEPCK promoter elements (e.g. AF1), the N-terminal activation domain of HNF4 (a.a. 1–24) is referred to as transactivation domain 1 (TAD1). This domain of HNF4 is described as AF1 in other papers. The C-terminal activation domain of HNF4, elsewhere referred to as AF2, is referred to as transactivation domain 2 (TAD2).

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Daryl K. Granner, MD, Department of Molecular Physiology and Biophysics,
Vanderbilt University Medical School, Nashville, TN 37232-0165 (USA)
Tel. +1 615 322 7004, Fax +1 615 322 7236, E-Mail daryl.granner@mcmail.vanderbilt.edu

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Role of the Hepatocyte Nuclear Factor Network in Maturity-Onset Diabetes of the Young (MODY)

Markus Stoffel

The Rockefeller University, Laboratory of Metabolic Diseases, New York, N.Y., USA

The MODY Gene Defects

Maturity-onset diabetes of the young (MODY) is a clinically and genetically heterogenous disease with an autosomal-dominant inheritance and an age of onset of 25 years or younger [1]. Using genetic linkage and candidate gene approaches, MODY genes have been localized to chromosomes 7, 12, 13, 19 and 20 [2–5] and are believed to be responsible for 2–5% of non-insulin-dependent diabetes mellitus (NIDDM). The gene on chromosome 7 (MODY2) encodes the glycolytic enzyme glucokinase which plays a key role in generating the metabolic signal for insulin secretion and in integrating hepatic glucose uptake [6–8]. The genes on chromosomes 20 (MODY1), 12 (MODY3), 19 (MODY5) and 13 (MODY4) are encoded by transcription factors hepatocyte nuclear factor-4α (HNF-4α), -1α (HNF-1α), -1β (HNF-1β, vHNF-1)) and homeobox transcription factor-1 (IPF-1, PDX-1, STF-1), respectively [9–12]. A significant portion ($\approx$20%) of MODY pedigrees do not show linkage to any of the known MODY loci, indicating that there is an unknown number of MODY genes that have yet to be identified (table 1).

Clinical studies indicate that MODY is associated with reduced and abnormal patterns of glucose-stimulated insulin secretion [13–15]. Subjects with glucokinase mutations usually have mild hyperglycemia that begins in early childhood and, in the absence of other risk factors such as obesity, has little tendency to progress. In contrast, patients with mutations in HNF-1α, HNF-1β and HNF-4α often develop diabetes after puberty. PDX-1 dependent diabetes (MODY4) has a relatively late disease onset ($\approx$35 years) but pedigrees show

Table 1. Genetic classification of early-onset type 2 diabetes (MODY)

Form of diabetes	Gene	Chromosome	Gene family
MODY1	HNF-4α	20q12	Orphan steroid hormone receptor
MODY2	GCK	7p13	Hexokinase IV
MODY3	HNF-1α	12q24.2	Homeodomain containing transcription factor
MODY4	PDX-1	13q12.1	Homeobox gene
MODY5	HNF-1β	17	Homeodomain containing transcription factor
MODYX	Unknown	Unknown	Unknown

autosomal dominant inheritance [11]. MODYs due to defects in the above transcription factors have more severe forms of hyperglycemia than glucokinase deficient patients and frequently develop complications.

Role of HNFs in Development, Differentiation and Metabolism

Hepatocyte nuclear factors (HNFs) are a diverse class of transcription factors which were initially identified by their binding activity to sites in the regulatory regions of genes that are expressed specifically in the liver [16]. Liver specific transcription depends on the binding of HNFs to distinct cis-acting sequences in gene control regions. The interactions between unique combinations of liver enriched transcription factors and ubiquitous transcription factors regulate and determine maximum transcription of a given target gene. This combinatorial control is an efficient mechanism to generate both diversity and stringency of tissue-specific gene expression [17–20].

Hepatocyte nuclear factors have been classified into five families including the homeodomain homolog HNF-1 (HNF-1α, HNF-1β), the basic leucine zipper protein family C/EBP, the forkhead homologue HNF-3 (HNF-3α, HNF-3β, HNF-3γ), the orphan steroid hormone receptor HNF-4 (HNF-4α, HNF-4γ), and a recently identified HNF-6 that has been shown to contain two different DNA-binding domains, a novel type of homeodomain and a homologue of the Drosophila cut domain [16, 21]. All of these transcription factors were found to be expressed not only in the liver, but also in the kidney, intestine, endocrine or exocrine pancreas, spleen and other tissues [21–23]. Hepatocyte nuclear factors regulate diverse biological processes such as development, differentiation and metabolism. Inactivation of mouse HNF-3β and HNF-4α by homologous recombination leads to defects in early embryonic development and death. HNF-3β null embryos do not form a distinct node

and lack a notochord indicating that HNF-3β has an essential role in the development of axial mesoderm in mouse embryos [24, 25]. Disruption of the HNF-4α gene leads to cell death in the embryonic ectoderm at 6.5 days and fail to undergo normal gastrulation [26]. Mutant mice lacking HNF-1α show normal development but develop a severe postnatal Fanconi-like syndrome and die at 3–4 weeks of age due to defects in liver, pancreatic β-cell and kidney function [27]. The effect of loss of function of HNF-1β on development, differentiation and metabolism is unknown.

The Transcriptional Network of Hepatocyte Nuclear Factors

Several lines of evidence suggest that a transcriptional hierarchy in which HNF4α regulates the expression of HNF-1α is responsible in both the determination and maintenance of the hepatic phenotype. The HNF-1β promoter contains evolutionarily conserved binding sites for transcription factors HNF-4α, AP1 and HNF-3. The presence of the HNF-4 binding site is crucial for HNF-1α activity both in vivo and in vitro [28–30]. Furthermore, stable expression of HNF-4α in a dedifferentiated rat hepatoma cell line has been shown to induce the reexpression of the endogenous HNF-1α gene [31]. Analysis of hepatocyte differentiation, using dedifferentiated hepatoma variants and intertypic cell hybrids which show extinction of all or most of the hepatic functions, has revealed that the abolition and reexpression of the hepatic phenotype correlates, respectively, with the disappearance of expression of HNF-4α and HNF-1α and with the reexpression of these two factors [32, 33]. Evidence that the functional hierarchy may also be important in pancreatic β-cell function comes from the finding that mutations in HNF-4α and HNF-1α are responsible for two clinically indistinguishable forms of early-onset type 2 diabetes, MODY1 and MODY3 [9, 10]. Furthermore, a point mutation in the HNF-4 binding site in the HNF-1α promotor can also result in the MODY3 phenotype [30].

HNF-3β has been shown to regulate the expression of STF-1/PDX-1 and to induce this transcription factor in pancreatic islet cells [35, 36]. Gene disruption studies in which the PDX-1 gene was targeted and inactivated have revealed that this factor serves as an important control switch for expression of both exocrine and endocrine pancreatic programs. In early pancreas development, PDX-1 is expressed in both exocrine and endocrine cell types, however, as pancreatic morphogenesis proceeds, PDX-1 expression is eventually restricted to β- and γ-cells of the islets, where it appears to regulate the expression of the insulin and somatostatin genes, respectively [37, 38]. The PDX-1 gene has also been shown to be essential for pancreatic development and normal pancreatic β-cell function in humans. Subjects with complete loss of PDX-1

function due to a homozygous mutation suffer from pancreatic agenesis and individuals with the same heterozygous mutation (P63fsdelC) develop early-onset type 2 diabetes (MODY5) [39, 40].

Molecular Hypothesis for Defects in MODY Transcription Factors

The finding that mutations in HNF-4α and HNF-α can cause early-onset type 2 diabetes suggests that this is a disorder of gene expression and that the HNF-regulatory network plays an important role in pancreatic islet function. Although these factors have been shown to play a significant role in the regulation of many genes expressed in the liver, very little information is available on HNF-4α and HNF-1α function in pancreatic β-cells. Based on our knowledge of HNF-4α and HNF-1α function in the liver, we hypothesized that abnormal expression of the HNF-transcriptional cascade and downstream targets leads to pancreatic β-cell dysfunction through the following mechanism(s) (fig. 1).

HNF-4α Is a Positive Regulator of HNF-1α Which May Act as a Weak Transcriptional Activator of Insulin

HNF-4α has been shown to be a positive regulator and activator of HNF-1α expression in the liver. Stable expression of HNF-4α in dedifferentiated rat hepatoma cells is able to activate the endogenous silent HNF-1α gene, thus indicating that a transcriptional hierarchy regulates HNF-4α and HNF-1α expression [32, 33]. HNF-1α has been shown to specifically bind to the FLAT-F element of the rat insulin I gene promotor and to act as a weak activator of insulin transcription [41]. Thus, a decrease in the amount of HNF-4α might have a critical effect on β-cell function by decreasing HNF-1α and insulin gene expression. However, it has not been shown that reduced HNF-4/HNF-1 levels lead to decreased transcription of the insulin gene in vivo.

HNF-4α Is a Key Regulator of Gene Products Involved in β-Cell Glucose Sensing, Glucose and Fatty Acid Metabolism

Pancreatic islets and hepatocytes appear to share a glucose sensing mechanism thought to involve glucokinase, a distinct form of hexokinase found in the hepatocyte, pancreatic β-cells and some neuroendocrine cells in the intestinal mucosa, and GLUT2, a member of the glucose transporter family found in the β-cell, liver, kidney and intestine [42–45]. The liver specific glucokinase promotor contains a sequence homologous to the HNF-1 consensus binding site and the GLUT2 promotor appears to contain putative HNF-1 binding sites [45]. We have shown that GLUT2 expression is decreased in the visceral endoderm of embryoid bodies of HNF-4α–/– ES-cells compared to HNF-4α+/+ ES-cells.

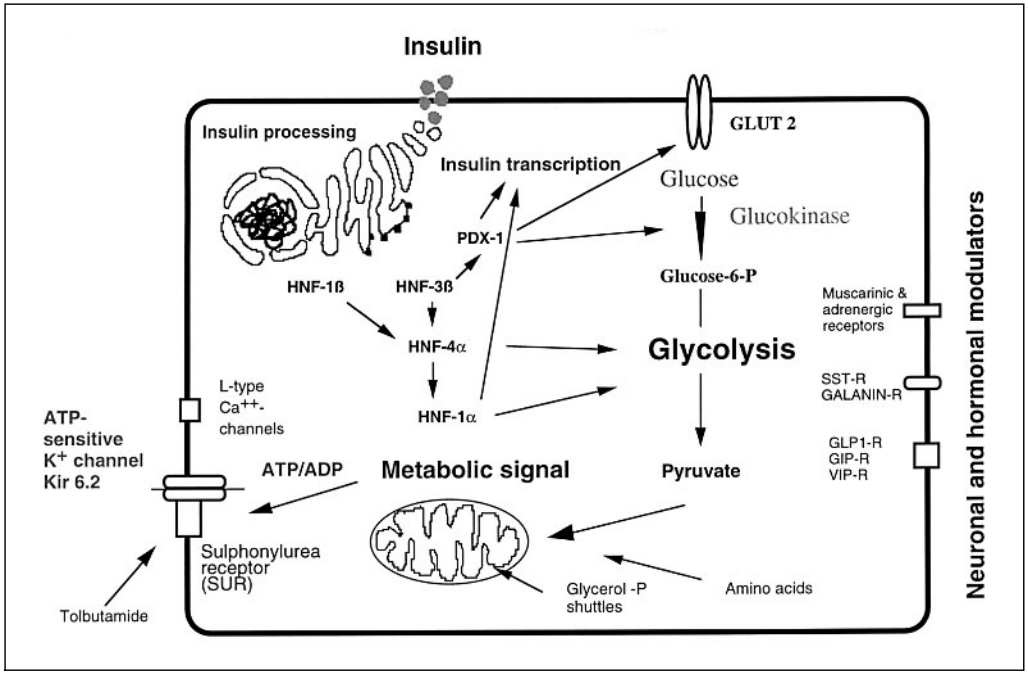

Fig. 1. Glucose-stimulated insulin secretion from pancreatic β-cells. Postprandial elevation of blood glucose leads to glucose transport into β-cells via glucose transporter-2 (Glut2), breakdown through the glycolytic pathway and mitochondrial oxidative phosphorylation. As a result, the ATP/ADP ratio increases, leading to an inhibition of ATP-sensitive, transmembrane potassium channels (Kir6.2) The change in membrane potential activates volatage-gated Ca^{2+} channels and results in a rise in cytosolic calcium levels that trigger fusion of prestored insulin vesicles with the plasma membrane and insulin release. The hepatocyte nuclear transcription factor network regulates the expression of enzymes of glycolysis, Glut-2 and the insulin gene. SST-R = Somatostatin receptors; GLP1-R = glucagon-like peptide-1 receptor; GIP-R = gastric inhibitory protein receptor; VIP-R = vasoactive inhibitory peptide receptor.

Thus, downregulation of key components of the glucose-sensing apparatus of the β-cells may be responsible for impaired insulin secretion and decreased glucose uptake by the liver in MODY1 and/or MODY3 [46]. Furthermore, we have shown that HNF-4α regulates three enzymes of glycolysis (L-pyruvate kinase, glyceraldehyde 3-phosphate dehydrogenase and aldolase-B) which could further impair glucose metabolism. The regulation of target gene expression can be either direct HNF-1-independent), mediated through reduced expression of HNF-1α or more likely is the result of a combination of activating actions of HNF-1α and HNF-4α as well as other transcription factors.

HNF-4α Is an Important Regulator of Genes Involved in Detoxification of Endogenous and Exogenous Substances

HNF-4α has been shown in vitro to play a role in regulating two cytochrome P450 genes (cyp2A4 and cyp4A6) [47]. Cytochrome P450 are phase I enzymes which are involved in lipid metabolism by oxidizing steroids, fatty acids, prostaglandins and other natural lipophilic compounds as well as drugs, carcinogens and other environmental contaminants. Downregulation of P450 genes might lead to intracellular accumulation of free fatty acids and metabolites that may lead to pancreatic β-cell damage. The concept of lipotoxicity has previously been proposed for obesity-dependent NIDDM [48], however, the HNF pathway has not been studied in animal models with this type of diabetes.

HNF-4α Is a Master Regulator of Genes Responsible for Cell Lineage Proliferation and Differentiation

The strong correlation between extinction and reappearance of HNF-4/HNF-1 binding activities and dedifferentiation and redifferentiation of the hepatic phenotype of hepatoma cell lines strongly imply that HNF-4α and HNF-1α play a crucial role in maintaining hepatocyte specific differentiation [32, 33]. Pancreatic β-cell lines, like hepatoma cells, can loose their phenotypic characteristics such as glucose induced insulin secretion. It is currently unknown if the HNF-pathway also plays a role in maintenance of the pancreatic β-cell phenotype and processes such as differentiation and regeneration.

Molecular Characterization of the HNF-Gene Mutations

MODY3 results from mutations in the HNF-1α gene and are the most common forms of MODY. More than 40 different mutations have been described in all exons of the gene. They include missense, nonsense, deletion, insertion and frameshift mutations. The molecular mechanism by which mutations in only one allele of the HNF-1α gene impair pancreatic β-cell function is unclear. HNF-1α can form homodimers or heterodimerize with the structurally related protein HNF-1β. Only HNF-1 dimers can bind to promoter elements and activate transcription. HNF-1α is composed of three functional domains: an amino-terminal dimerization domain (amino acids 1–32), a DNA-binding domain with POU-like and homeodomain-like motifs (amino acids 150–280), and a COOH-terminal transactivation domain (amino acids 281–631). Most HNF-1α mutants including defects in the HNF-1α promoter or in the dimerization domain can be predicted to result in loss of function. However, mutant HNF-1α proteins with an intact dimerization surface may impair pancreatic

β-cell function by forming nonproductive dimers with wild-type protein, hereby inhibiting its activity. This mechanism has been shown for frameshift mutation HNF-1α P291fsinsC, a common mutation in MODY3 patients. P291fsinsC-HNF-1α showed no transcriptional transactivation activity in HeLa cells, which lack endogenous HNF-1α. Overexpression of HNF-1α(P291fsinsC) in MIN6 cells, a mouse β-cell line, resulted in an approximately 40% inhibition of the endogenous HNF-1α activity in a dosage-dependent manner. Furthermore, heterodimer formation between wild-type and P291fsinsC mutant proteins were observed by electrophoretic mobility shift assay. These data suggest that the HNF-1α(P291fsinsC) mutant protein may have dominant-negative activity [49].

The first mutation associated with diabetes that was identified in the MODY1 gene HNF-4α was found to be a nonsense mutation in codon 268, Q268X [9]. This mutation generates a truncated protein containing an intact DNA-binding domain but lacking part of the AF2 region. To investigate the mechanism by which mutated HNF-4α can lead to diabetes, we first studied the functional properties of the MODY1 mutant protein. We showed that it has lost its transcriptional transactivation activity, fails to homo- or heterodimerize and bind to DNA implying that the MODY1 phenotype is due to a loss of HNF-4α function [46]. This implies that a haploinsufficiency of HNF-4α can lead to the MODY1 phenotype. Six additional HNF-4α mutations have been associated with type 2 diabetic: K99fsdelAA, R154X , R127W, V255M, E276Q and V393I [50–56]. Deletion mutant K99fsdelAA leads to a frameshift and truncated protein. Nonsense mutation R154X produces a truncated protein containing only the DNA-binding domain and the AF1 transactivation domain. These proteins can be predicted to lack transcriptional transactivation activity. The missense mutation V393I located in the AF2 domain has been shown to lead to a modest decrease in transactivation potential. Three point mutations, R127W, V255M and E276Q, which were identified in Japanese, Danish and UK families, respectively, have not been characterized. HNF4(R127W) is located between the DNA binding and AF2 domain and HNF4(V255M) and HNF4(E276Q) are positioned within the AF2 region. We have recently demonstrated that HNF4(E276Q) is a loss of function mutation and that HNF4(R127W) and HNF(V255M) are functionally normal and most likely represent rare polymorphisms [63].

HNF4-Regulated Gene Expression

The identification of target genes that are regulated by hepatocyte nuclear factors and their functional analysis are powerful approaches to study the molecular pathogenesis of MODY. We have taken a genetic approach using

genetically manipulated embryonic stem (ES) cells to study the effect of loss of HNF-4α function on target gene expression [46]. HNF-4α null mice die during development prior to formation of the pancreas and so cannot be used to identify HNF-4α regulated genes in the pancreatic β-cells. However, the visceral endoderm (VE), which expresses HNF-4α and its target genes, displays many characteristics of both the liver and endocrine pancreas and was used as a model to sudy HNF-4α regulated gene expression. This system has the advantage that it studies gene regulation of any target gene in a chromosomal context (in native chromatin) and at physiological expression levels of HNF-4α. Furthermore, the VE is derived from euploid ES-cells which do not have the drawbacks of transformed, genetically abnormal cell lines that are frequently used in in vitro studies. ES-cells can be grown in suspension in the absence of LIF and can be differentiated to form embryoid bodies (EBs) which contain VE . The VE expresses many genes which regulate glucose metabolism including, for example, GLUT2, aldolase B and L-pyruvate kinase. Inactivation of the HNF-4α gene by targeted disruption and homologous recombination in ES-cells allowed us to test if loss of function of HNF-4α leads to an impairment of gene expression of key components of the glucose-stimulated insulin secretion signaling pathway in vitro. Gene expression in differentiated wild-type, heterozygous and null HNF-4α (HNF-4a+/+, +/–, –/–) embryoid bodies was assayed by quantitative RT-PCR. EBs produced from three HNF-4α null ES-cell lines did not express HNF-4α mRNA. Steady-state mRNA levels of visceral endoderm markers Gata-4 and HNF-1β were also measured to demonstrate that each EB preparation had similar amounts of visceral endoderm. In order to identify downstream target genes of HNF-4α we studied the expression of 100 genes which are known to have essential functions in insulin secretion in the pancreatic islet, and glucose metabolism in the liver. Genes tested in the expression screen included glucose transporters, enzymes of glucose and fatty acid metabolism, intracellular storage proteins, ion channels and G-protein coupled receptors of the seven transmembrane spanning superfamily, hormones and transcription factors. Four genes involved in glucose transport and metabolism (glucose transporter 2 (GLUT2), aldolase B (aldoB), glyceraldehyde-3-phosphate dehydrogenase (1,3 BGD) and L-pyruvate kinase (L-PK) were significantly reduced or absent. This result also held true in vivo when we collected E.8.5 embryos from crosses of HNF-4+/– mice and assayed VE gene expression in normal or HNF-4α mutant mice. Expression analyses of target genes in embryos of HNF-4α null embryos confirmed the results obtained from the ES-cell lines and suggests that downregulation of these genes in vivo may be even more dramatic than in vitro [46]. The impairment of glucose utilization and mitochondrial oxidation due to reduced gene expression of glucose transporter 2 and several glycolytic enzymes

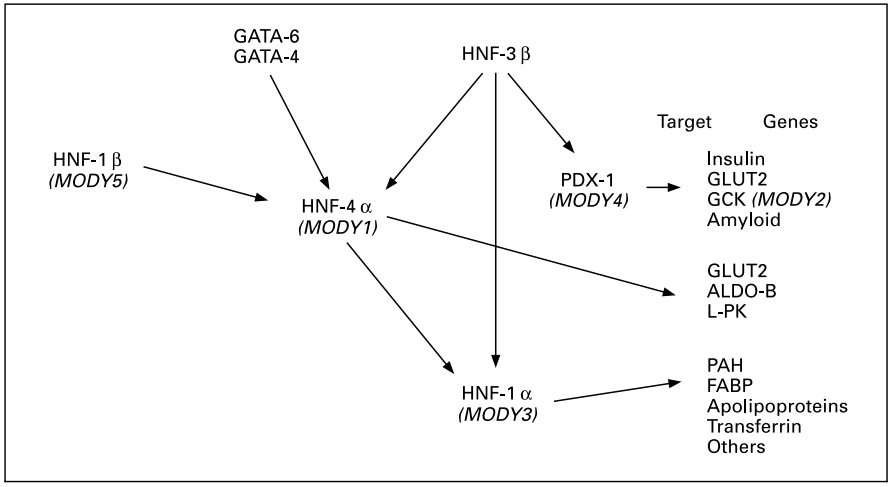

Fig. 2. The hepatocyte nuclear factor network and their target genes. Transcriptional hierarchy of hepatocyte nuclear factors. Arrows indicate transcriptional activation. GCK = Glucokinase; GLUT2 = glucose transporter 2; ALDO-B = aldolase B, L-PK = pyruvate kinase (liver isoform); PAH = phenylalanine hydroxylase.

suggest a molecular basis for the diabetic phenotype of MODY1 patients (fig. 2).

A similar pathomechanism was subsequently proposed for patients with HNF-1α mutations [57]. Graded overexpression of HNF-1α or controlled overexpression of a dominant negative form of HNF-1α was able to increase or diminish, respectively, the expression of insulin gene, L-pyruvate kinase and glucose transporter 2 genes in INS-1 cells [57]. Further evidence for a metabolic defect involving glycolytic flux comes from studies in mice lacking the HNF-1α gene. HNF-1α null mice have impaired insulin secretion and intracellular calcium ($[Ca^{2+}]_i$) responses following stimulation with nutrient secretagogues such as glucose and glyceraldehyde but display normal responses with non-nutrient stimuli such as potassium chloride. Flux of glucose through glycolysis in islets from mutant mice is reduced which results in an impairment of ATP generation in response to glucose. Patch clamp recordings show that ATP-sensitive K^+ currents (KATP) in beta-cells are insensitive to suppression by glucose but respond normally sensitive to ATP. Furthermore, exposure of HNF-1α null islets to mitochondrial substrates suppressed KATP, elevated $[Ca^{2+}]_i$, and corrected the insulin secretion defect. These results provide strong evidence that hepatocyte nuclear factor-1alpha diabetes also results at least in part from defective beta-cell glycolytic signaling [58].

Upstream Regulators of HNF-4α

We have shown that loss of function of HNF-4α can lead to MODY1 and that HNF-4α is a key regulator of glucose transport and metabolism in vivo [46]. This implies that upstream regulators of HNF-4α expression may also be critical for controlling these pathways. We studied the role of the HNF-3 gene family on regulation of HNF-4α expression because it has previously been shown that HNF-4 promoter sequences contain evolutionarily conserved HNF-3-binding sites [34]. Furthermore, all HNF3 members are expressed in pancreatic islets and β-cell line MIN6 [22].

To study HNF3-dependent gene expression we generated HNF-3α and HNF-3β null ES-cells. We measured gene expression by RT-PCR in day 21 HNF-3β+/+, +/– and –/– EBs. The expression of HNF-4α was markedly reduced in HNF-3β–/– EBs, as were several genes known to be in vivo targets of HNF-4α [46, 59]. These genes included apolipoproteins AI, AII, AIV, B, CII and CIII, transthyretin, aldolase B, and L-pyruvate kinase. As expected, expression of HNF-1α was also reduced in the absence of HNF-3β. HNF-3α mRNA was absent in HNF-3β null EBs, implying that HNF-3β is absolutely required for HNF-3α expression. These results demonstrated that HNF-3β is a major regulator of HNF-4α gene expression and the master regulator of the hepatocyte nuclear factor transcriptional network [60] (fig. 2).

GATA6 has also recently been shown to regulate HN-4α gene expression during embryonic development and to transactivate the HNF-4α promoter in vitro (fig. 2). GATA6 belongs to a family of zinc finger transcription factors and is expressed in the extraembryonic and embryonic endoderm during early development. Later, GATA6, together with GATA4 and GATA5, is expressed in the embryonic heart and gut epithelium. The expression levels of GATA6 in pancreatic islets of adult mice are very low and it is unclear if GATA6 is an important regulator of HNF-4α gene expression in differentiated pancreatic β-cells (M. Stoffel, unpublished).

The HNF-Transcriptional Network and Their Target Genes are Regulated by Insulin

The strong regulation of metabolically controlled genes such as enzymes of glycolysis by HNF-3 and HNF-4α led us to investigate if HNF-3 and HNF-4α gene expression are affected by insulin. We measured steady-state levels of HNF-3α, a physiological repressor of gene transcription in the visceral endoderm of embryonic bodies [60], and HNF-3β mRNAs in day 14 EBs which were cultured for 24 h in serum free media containing 0, 5 or 50 n*M*

insulin and 20 m*M* glucose. In the presence of insulin HNF-3β expression was upregulated whereas HNF-3α was reduced. Moreover, downstream targets of HNF-regulation, such as aldolase B and L-pyruvate kinase, were also significantly increased. These data show a striking correlation between the presence of insulin, HNF-3α:HNF-3β ratios and expression of downstream targets. From this we concluded that insulin is a positive modulator of the HNF-transcription factor network in EBs [60]. It has recently been shown that targeted disruption of the insulin receptor substrate-2 gene by homologous recombination in mice leads to defects in both insulin secretion and insulin action [55]. Surprisingly, mice lacking IRS-2 lost the ability of β-cell compensation for insulin resistance. Furthermore, mice with a pancreatic β-cell selective inactivation of the insulin receptor gene also exhibit defects in insulin secretion [62]. Future studies will show if the pancreatic β-cell defect in mice with impaired insulin signaling is caused by an impairment of gene expression of the hepatocyte nuclear factor network and their downstream target genes.

Conclusion

The majority of early-onset forms of type 2 diabetes are caused by genetic defects in transcription factors that lead to an impairment of gene expression essential for normal functioning of pancreatic β-cells. These MODY genes are functionally related and form part of an integral network of transcription factors that regulate gene expression of target genes including insulin, glucose transporter and metabolic enzymes. The molecular and physiological characterization of this network as well as the identification of novel target genes will provide further insights into essential pathways for normal β-cell function as well as pathophysiological mechanisms in type 2 diabetes.

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Markus Stoffel, MD, Laboratory of Metabolic Diseases, Box 292, 1230 York Avenue,
New York, NY 10021 (USA)
Tel. +1 212 327 8797, Fax +1 212 327 7997, E-Mail Stoffel@rockvax.rockefeller.edu

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