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MAMMALIAN GLUCOKINASE

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INTRODUCTION

Glucose transport and phosphorylation are the initial events in glucose utilization by all tissues and cell types. The necessary first step in glucose utilization, the movement of the molecule across the plasma membrane, is accomplished by a family of glucose transporters, designated GLUT 1–5, that operate by facilitated diffusion to move glucose down a high to low concentration gradient (10, 78, 85). Glucose uptake occurs when the

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concentration of extracellular glucose exceeds the intracellular concentration, thus favoring glucose entry. This is generally the case, a notable exception being hepatocytes that export glucose when they are actively engaged in glycogenolysis or gluconeogenesis, or intestinal and renal cells that are involved in the trans-epithelial movement of glucose. This downhill concentration gradient is maintained by the conversion of glucose to its phosphate ester, glucose-6-phosphate (G-6-P), which cannot exit the cell.

In mammalian cells the phosphorylation of glucose to G-6-P is catalyzed by a family of closely related enzymes, the hexokinases. Four mammalian hexokinases have been characterized (38, 55). These are designated hexokinase (HK) I, II, III, and IV, according to their relative mobility following starch gel electrophoresis, or, less commonly, A, B, C, and D, respectively, according to their order of elution from DEAE-cellulose (119). HK I–III have several properties in common, including a molecular weight of ~100 kd, the ability to phosphorylate several hexoses, and a relatively high affinity for glucose. In addition they are inhibited by physiologic concentrations of G-6-P, the product of the reaction when glucose is the substrate. Although generally similar, each of these hexokinases has a unique set of kinetic properties and a different pattern of tissue distribution (38, 55, 119). One family member, HK IV, more commonly known as glucokinase (GK), is structurally and functionally different from the others, and is the subject of this review. Some features that distinguish GK from HK I–III include a mass approximately half that of the other family members, a lower affinity for glucose, a much lower affinity for other hexoses, and the lack of significant feedback inhibition by physiologic levels of G-6-P [see Table 1 for comparison; and see (128)]. The significance of these differences in the function of GK in the hepatocyte and the β cell of pancreatic islets is a subject of this review, as are recent observations concerning the structure and regulation of the GK mRNAs in these cells. The structure of the GK gene, and the apparent role of this gene in some forms of noninsulin-dependent diabetes, are discussed. We have attempted to build upon the excellent review by Weinhouse (128) by emphasizing observations made in the past few years, and we apologize in advance for omissions of data and references that were caused by space limitations.

Discovery of Glucokinase

Glucose phosphorylating activity in mammalian cells was originally referred to as "hexokinase," but it soon became apparent that there were tissue differences not readily understandable on the basis of a single enzyme. For example, a complex situation was noted in liver. A hexokinase that had the typical low K_m for glucose, and that accepted several hexoses as substrates,

Table 1 A comparison of glucokinase with other hexokinases[a]

	GK	HK I-III
K_m glucose	5–12 mM[b]	0.02–0.13 mM
K_m ATP	~0.5 mM	0.2–0.5 mM
K_i G-6-P	60 mM	0.2–0.9 mM
Molecular weight	52 kd	~100 kd
Substrate preference		
Glucose	1[c]	1[c]
Mannose	0.8	1–1.2
2-Deoxyglucose	0.4	1–1.4
Fructose	0.2	1.1–1.3

[a] Several features of rat glucokinase are compared to rat hexokinases I-III (119, 128). Properties of the human isoforms are similar to those of the rat counterparts.

[b] This is an approximate K_m of the purified enzyme. The exact K_m cannot be determined because of the positive cooperativity described in the text. The enzyme in homogenates of liver cells has a higher K_m owing to the presence of the regulatory system described in the text.

[c] The activity of glucose as a substrate is taken as 1. The other numbers are expressed in relation to the activity of glucose as a substrate.

was discovered first (103). A second hexokinase, which had a high K_m for glucose oxidation to CO_2, was later identified in liver slices (13). This activity was dependent upon insulin, since it was reduced in diabetic animals, and was restored to normal with insulin treatment (105). Subsequent experiments in liver homogenates confirmed the presence of a new hexokinase that had a high K_m for glucose (10 mM) (100). This activity was named glucokinase because it had a greater selectivity for glucose, and it was given a unique number by the Enzyme Commission (EC 2.7.1.2). It is now generally accepted that GK is a member of a family of enzymes that catalyze the same general reaction; thus HK I–IV are collectively given the same designation (ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1), as discussed by Cornish-Bowden (21). The names hexokinase IV and glucokinase can both be justified, but for the purposes of this review we prefer to use glucokinase, the more common designation.

An important feature of GK is its cell-specific distribution and regulation (49, 65, 72, 92, 127). The enzyme is primarily found in hepatic parenchymal cells and in the β cells of the pancreatic islets. The previously mentioned kinetic and regulatory features that distinguish GK from HK I–III, and the presence of the enzyme in the liver and the β cells of the pancreatic islets,

have physiologic importance. These tissues are fed by the portal vein, in which postprandial glucose concentrations reach high levels; therefore, GK is in position to play a key role in glucose homeostasis.

As mentioned above, at least five glucose transporters are involved in the movement of glucose across the plasma membrane of cells. A high K_m transporter (GLUT 2) is found in hepatocytes and β cells, which predominantly express GK, the high K_m hexokinase. The general association of a particular hexokinase with a specific glucose transporter has also been observed in the case of the lower K_m hexokinases. For instance, the lowest K_m transporter is generally associated with the lowest K_m hexokinase (GLUT 1 with HK I), and the intermediate K_m transporter with the corresponding intermediate K_m hexokinase (GLUT 4 with HK II). The reason(s) for these associations has not been completely established, but the combination of GLUT 4 and HK II appears to have a physiologic explanation. Both of these proteins are found in tissues in which insulin increases glucose uptake and utilization (10, 38, 55, 78, 85), and their coordinate regulation by insulin is probably of central importance to these processes. Similarly, the combination of GLUT 2 and GK (HK IV) is also involved in glucose homeostasis, as described below. GK is regulated by insulin in liver and by glucose in β cells (9), and GLUT 2, although not regulated by insulin, is at least indirectly involved in the secretion of insulin (74, 118).

The Glucokinase Reaction

Glucokinase catalyzes the reaction: Glucose + Mg•ATP → G-6-P + ADP. Attempts to purify GK began shortly after the discovery of the enzyme, but were complicated by the fact that the enzyme is not present in high concentration in either liver or β cells (and the latter are themselves not abundant), and it is more sensitive to denaturation and proteolysis than are the other hexokinases (86). Extensive purification with a yield suitable for peptide isolation and amino acid sequencing was accomplished only very recently (3). GK comprises about 0.005% of total protein in liver, so a 21,000-fold purification was required to obtain homogeneous enzyme (3). If GK is 20 times less abundant in β cells (49), a 400,000-fold purification would be required to obtain homogeneous enzyme from this source; needless to say, this has not been accomplished. The purified hepatic enzyme has a specific activity of ~180 U/mg [1 unit (U) of GK activity catalyzes the production of 1 μmol of G-6-P in 1 min at 32C], and it preferentially catalyzes hexose phosphorylation in the order: glucose>mannose>2-deoxyglucose> fructose [Table 1; (3, 119, 128)]. GK-activity is strongly inhibited by glucosamine and its derivatives, by mannoheptulose, and by alloxan (58, 128).

Glucokinase shows cooperative dependence (sigmoidal kinetics) with

respect to the glucose concentration. This cooperativity increases with the Mg•ATP concentration, but the interaction of the latter with GK is itself not cooperative (108). A Hill coefficient of ~1.5 supports the observation that glucose binds to GK with positive cooperativity (108, 109). Cooperative interactions between subunits of an enzyme are frequently observed, but this kinetic behavior is unusual in monomeric enzymes such as GK (93). In the latter cases, this behavior is best explained on the basis of a "mnemonical" model in which the enzyme exhibits a "memory" phenomenon (109). According to this model, the enzyme exists in two conformations that are not in equilibrium with each other under steady-state conditions. Substrate (glucose) binds with differential affinity to the two enzyme forms, and an essential feature of the "mnemonical" model is that the enzyme relaxes relatively slowly after release or transformation of substrate. Enzymes like GK "remember" the high affinity conformation caused by their interaction with substrate, and in this state binding of the next substrate molecule proceeds more rapidly. The non-Michaelian or cooperative regulatory behavior is basically a consequence of the different affinity of two conformations of enzyme for substrate, e.g. glucokinase for glucose (89).

There is little GK activity below 2.5 mM glucose, and positive cooperativity is most apparent at glucose concentrations around 5 mM (108). Since transport is not thought to be rate-limiting, small elevations of the blood glucose above the normal fasting level of 5 mM result in rather large increases in GK activity and enhanced glucose phosphorylation. Glucose in the concentration range of 5–10 mM therefore has a greater effect on the activity of the enzyme than would occur in a system that operates on the basis of pure Michaelis-Menton kinetics. These observations regarding positive cooperativity apply to both the hepatic and β-cell enzyme (72).

The conformational changes predicted by the "mnemonical" model are supported by the X-ray crystal structures obtained for yeast hexokinase. This enzyme, like GK, is a 50-kd protein that is not subject to feedback inhibition by G-6-P (106). The structural similarities between yeast HK and GK make it likely that similar reaction mechanisms are employed by the two enzymes. Hexokinases contain two lobes separated by a deep cleft, and all undergo significant changes upon the binding of substrate into this cleft (1). Yeast hexokinase, with glucose bound, has a very different conformation from the enzyme without bound substrate (1, 12). Glucose binds to hexokinase in a deep cleft that closes to envelop the substrate and to orient catalytic groups and/or to exclude solvent. Glucose analogs with substitutions on the 2-carbon position are competitive inhibitors of glucose binding, but do not serve as substrates. These analogs prevent the lobes of the enzyme from approximating one another, so it is inferred that a glucose-induced conformational change is essential for catalysis.

In the case of yeast hexokinase, the carboxylate group of an aspartic acid residue (Asp^{211}) forms a hydrogen bond with the phosphoryl acceptor (the hydroxyl group on the 6-carbon position) and probably functions as a general base catalyst (1). Removal of solvent from this region of the enzyme is thought to enhance nucleophilicity and favor catalysis (1). The amino acids Ser^{158}, Asn^{210}, Asp^{211}, Glu^{269}, and Glu^{302} in yeast hexokinase are presumed to form hydrogen-bonds with the hydroxyl groups of glucose (75). These amino acids are conserved in GK as Ser^{151}, Asn^{204}, Asp^{205}, Glu^{256}, and Glu^{290}, respectively, so the binding of glucose to GK is probably by a mechanism similar to that employed by yeast HK (3). Asp^{205} in GK is certainly important for activity, as mutation of this residue to alanine results in an enzyme that has one five-hundreth the activity of the wild-type GK (57).

The residues involved in ATP binding have not been defined as precisely as those involved in glucose binding. A putative ATP-binding domain in mammalian hexokinase was identified based on the conservation of amino acid sequences between known hexokinases and the ATP-binding region found in protein kinases (3). A lysine located 11–14 residues from the putative core binding sequence, corresponding to Lys^{111} in yeast HK, appears to be involved. A role of Lys^{111} in ATP-binding in yeast HK was suggested by the observation that this residue covalently binds the ATP affinity label pyridoxyl 5′-diphospho-5′-adenosine (111). A 50 amino acid peptide from yeast HK, containing the predicted ATP-binding domain and some flanking sequence (Glu^{78} to Leu^{127}), also binds 2′, (3′)-0-(2,4,6-trinitrophenyl) adenosine-5′-triphosphate, an ATP analog that is a weak substrate for hexokinase. This provides further evidence that this domain is part of the catalytic site (6). Lys^{111} in yeast HK corresponds to Lys^{558} in mammalian HK I. Mutation of Lys^{558} to arginine results in a 30% decrease of the V_{max} of HK I, and mutation to methionine results in a 71% reduction (5). These mutations have no effect on the K_m for ATP (or glucose); thus Lys^{558} cannot be assigned a direct role in ATP-binding (5). No direct testing of the predicted ATP-binding site of GK, or the role of the invariant lysine, Lys^{102}, has been reported to date. More definitive information about the specific sites required for catalysis will be forthcoming as GK molecules with specific mutations are analyzed, and when the crystal structure of the protein is determined.

Regulation of Glucokinase Activity

Physiologic concentrations of G-6-P, the product of the glucokinase reaction, do not inhibit the enzyme, but more distal products of glucose and lipid metabolism can affect activity. G-6-P, in addition to being a substrate for glycogen synthesis, is also a substrate for fatty acid synthesis through the glycolytic and pentose phosphate pathways. Elevated levels of long-chain acyl-CoAs, such as palmitoyl CoA, reversibly inhibit GK from several

species, and would therefore indirectly retard fatty acid synthesis by feedback inhibition of GK (116, 117). There is no indication that the micromolar concentrations of free, long-chain acyl-CoAs required for this inhibition of GK are ever achieved in cells; hence the physiologic relevance of this effect is questionable.

The short-term regulation of HK I–III activity is accomplished by G-6-P, and thus by the pathways that use this compound as a substrate. Glucose, through the "mnemonic" mechanism described above, provides short-term regulation of GK. Recent experiments reveal an additional complex and important mechanism for regulating GK activity (124). The original observation was that fructose increased the rate of glucose phosphorylation in isolated hepatocytes (18). The effect of fructose, which occurred at low, physiologic concentrations (~50 μM), was to decrease the K_m for glucose from the 15–20 mM range, typically seen in crude cell homogentates, to the 5–10 mM range seen when pure enzyme is assayed (18, 123). The effect of fructose was exerted within a few minutes and was lost if this hexose was removed from the incubation medium. The observation that this stimulatory effect was mimicked by sorbitol and D-glyceraldehyde, but not by glycerol or dihydroxyacetone, led Van Schaftingen and Vandercammen to postulate that fructose-1-phosphate (F-1-P) was the mediator of this positive effect on GK (25, 123).

F-1-P did stimulate glucose phosphorylation in crude extracts of liver cells, but it was ineffective when tested against GK that had been partially purified by passage over an anion-exchange chromatography column. It should be noted, in this context, that GK elutes from such columns at a relatively high salt concentration. Aliquots of column fractions that eluted at a lower salt concentration directly inhibited purified GK and restored the ability of F-1-P to stimulate glucose phosphorylation. This observation led to the isolation of a regulatory protein which, upon further purification, also became ineffective. This was due to the removal of phosphoglucoisomerase, the enzyme that catalyzes the interconversion of G-6-P and fructose-6-phosphate (F-6-P). The requirement of the regulatory protein for F-6-P was formulated on the basis of this experiment, since G-6-P has no effect on GK (122).

This regulatory system therefore consists of F-1-P, F-6-P, and a protein of ~62 kd (Figure 1). F-6-P is half-maximally effective at a concentration of ~10 μM, and it interacts competitively with F-1-P. In the presence of F-6-P the regulatory protein (which has no enzymatic activity itself) forms a reversible, heterodimeric complex with GK [Figure 1; (120)]. This interaction results in competitive inhibition of GK with respect to glucose. Complex formation, and the inhibition of GK activity, is competitively prevented by F-1-P (Figure 1). The regulatory protein and palmitoyl CoA, another competitive inhibitor of glucose binding to GK, apparently bind to the same site. These compounds do not have additive effects, but both are synergistic

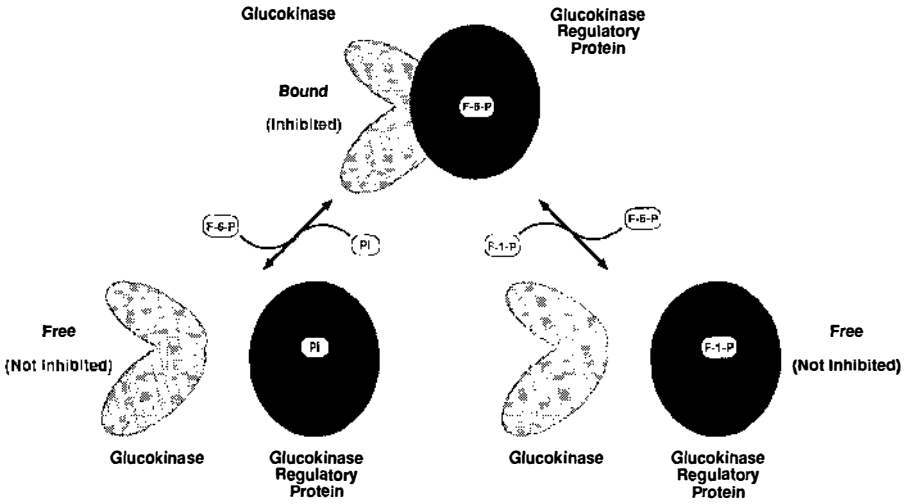

Figure 1 Proposed model for the regulation of glucokinase activity. The affinity of GK for glucose is changed by its interaction with the regulatory protein. The regulatory protein binds to GK allosterically and inhibits GK by decreasing the apparent affinity of the enzyme for glucose. The regulatory protein with fructose-6-phosphate (F-6-P) bound is in a conformation capable of interacting with, and inhibiting, GK. Fructose-1-phosphate (F-1-P) or inorganic phosphate (P_i) competes with F-6-P for binding to the regulatory protein. Regulatory protein with F-1-P or P_i bound is in a conformation that is not capable of interacting with GK, thus GK is not inhibited.

with competitive inhibitors, such as *N*-acetyl-glucosamine, that bind to the catalytic site. It is therefore presumed that the regulatory protein binds to an allosteric site and that this interaction alters the affinity of the catalytic site for glucose.

Sorbitol-6-P, a F-6-P analog, inhibits GK by binding to the regulatory protein (27). This interaction is probably not of physiologic relevance, but it emphasizes the important role of F-6-P. Several compounds normally found in hepatocytes serve as activators, but of these only inorganic phosphate (P_i) is effective at concentrations achieved in the hepatocyte (27, 121). P_i, like F-1-P, is thought to prevent the interaction of the regulatory protein with GK (Figure 1).

The discovery of the regulatory protein provides an explanation for the previously enigmatic observations that (*a*) the apparent K_m of GK for glucose is higher in intact hepatocytes than it is with the pure enzyme, and (*b*) dietary fructose increases glucose utilization. The latter observation merits additional observation. Since there is no fructose in the portal vein during the post-absorptive state, there is no fructose in the hepatocyte, and no F-1-P. F-6-P levels in the hepatocyte are, however, in the 10–50 μM range, which

is sufficient to bind to the regulatory protein. Under these circumstances the regulatory protein will bind to GK and raise the K_m for glucose of the enzyme. The ingestion of fructose, a constituent of most foods of vegetal origin, results in increased F-1-P levels in the hepatocyte, a decreased ability of the regulatory protein to bind to GK, and a decreased K_m (higher affinity) for glucose. In a direct test, fructose administration did increase glucokinase flux; thus this hexose could be a dietary signal for increased hepatic glucose uptake (123).

In summary, a regulatory protein binds F-6-P and this complex binds to, and inhibits, GK (see Figure 1). Although the regulatory protein is present in amounts sufficient to inhibit all GK activity in an hepatic cell, this inhibition is incomplete (~70%), presumably because P_i interferes with the interaction. F-1-P also interferes with the binding of F-6-P to the regulatory protein, and thus with the interaction of the latter with GK. F-1-P therefore causes a reduction of the K_m for glucose of GK in an intact cell or cell homogenate. The regulatory protein inhibits GK from many species, but has no effect on mammalian HK I–III, on yeast HK, or on bacterial GK (121). The regulatory protein is also found in the β cell, but the role of this protein in the regulation of GK in this cell type has not been established (68).

GLUCOKINASE GENE, mRNA, AND PROTEIN

In the past five years the techniques of molecular biology have been applied, with remarkable success, to the study of GK. The isolation of a cDNA for hepatic GK directly led to the isolation of the GK gene and of the islet cell GK cDNA. The initial studies were accomplished using rat tissues, but the human cDNAs and gene were quickly obtained. These observations played a key role in the discovery that mutations in the GK gene are associated with a form of noninsulin dependent diabetes mellitus (discussed below).

Hepatic Glucokinase mRNA and Protein

The independent cloning of hepatic GK cDNAs by two groups was the first step in the molecular genetic analysis of GK. A liver cDNA library was the obvious choice for the cloning of GK cDNA. The concentration of the enzyme in liver is greater than in β cells, the liver has a much larger mass, and the abundance of hepatic GK mRNA can be induced by dietary manipulation, hence there are more copies of GK cDNA in a library made from an hepatic source. Iynedjian et al utilized a GK antibody to screen an hepatic cDNA expression library. The cDNA isolated was used to show the expected regulation of hepatic GK mRNA by a variety of nutritional and hormonal stimuli. Based on these observations, and hybrid-selected translation of the mRNA, Iynedjian et al inferred that this was an authentic GK cDNA (51).

Andreone et al used a different approach (3). They purified hepatic GK to homogeneity, obtained amino acid sequence information for about half the protein, and then used this sequence to design oligonucleotide probes that were employed to isolate several hepatic GK cDNAs (3). Andreone et al deduced the complete sequence of hepatic GK from the cDNA sequence information and found it to be a 465 amino acid polypeptide with a mass of 51,924 daltons and a pI of 4.85 (3). All of the peptides sequenced from the purified GK were found in the sequence deduced from the cDNA, thus providing direct evidence of the authenticity of this GK cDNA. Putative glucose and ATP-binding domains (see above) were identified in the protein based on their similarity to domains found in yeast HK and mammalian HK I (3). The enzyme is very similar to the other known HK family members. The amino acid sequence of GK is 33% identical to yeast HK, 53% identical to the carboxy-terminal portion of the rat HK I, and 55% identical to the carboxy-terminal portion of rat HK II.

Rat Glucokinase Gene

The availability of the hepatic GK cDNA directly led to the isolation of the GK gene. A Southern blot analysis of rat genomic DNA was consistent with the presence of a single GK gene. Magnuson et al used the hepatic GK cDNA clone to isolate overlapping bacteriophage DNAs that contained all the exons encoding the hepatic GK mRNA (66). Ten exons (Figure 2*A*), spanning 15.5 kilobases (kb), were identified and the transcription initiation site was identified, as discussed below.

β-*Cell Glucokinase mRNA and Protein*

The availability of the hepatic GK cDNA also led to the isolation of GK cDNAs from libraries derived from cells of pancreatic β-cell origin. The analysis of these cDNAs led to some unexpected conclusions. Two groups found that the GK mRNAs in islet and insulinoma tissues are approximately 200 nucleotides (nt) longer than the liver mRNA (~2600 nt versus ~2400 nt) (50, 67). A comparison of the hepatic and insulinoma GK cDNAs revealed that the variation in the length is due to a difference at the 5′ end of the mRNAs (67). The length of the open reading frame is the same in mRNAs produced in both tissues, but 11 of the first 15 amino acids encoded are different. The sequence of the remainder of the enzyme is identical (46, 66). These changes have a very small effect on the mass and pI of the enzyme, and the kinetic and physical characteristics of GK in the liver and pancreatic β cells are virtually indistinguishable (49, 72). This is probably why the existence of different isoforms was not suspected by biochemical and immunologic techniques.

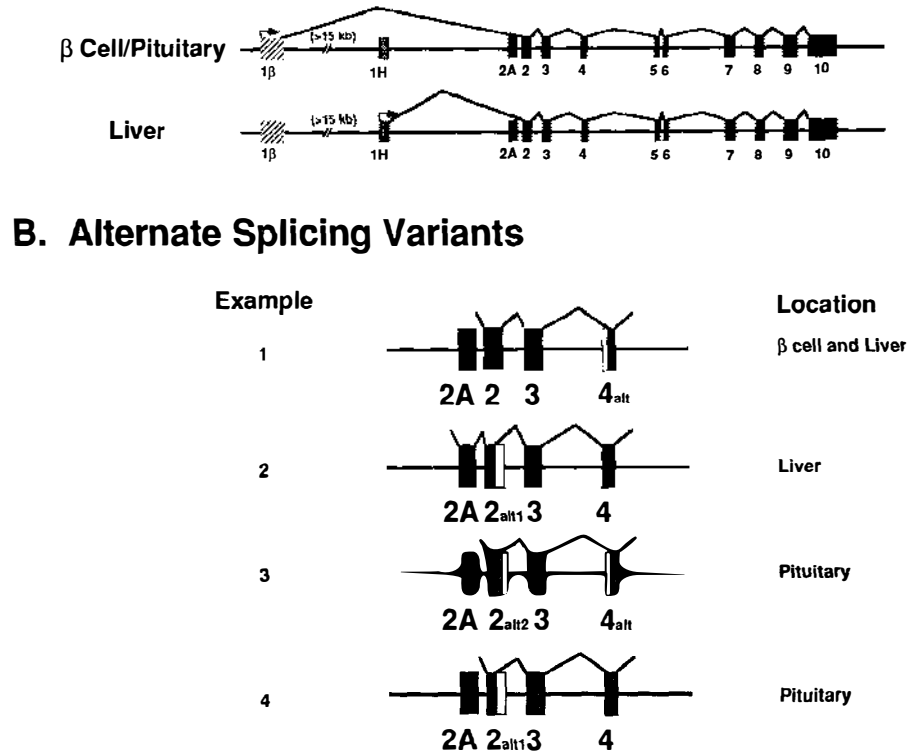

Figure 2 A schematic structure of the glucokinase gene showing alternate transcription units and splicing variants. Multiple glucokinase mRNAs are produced from a single gene through the use of alternate promoters and alternate RNA processing. Panel *A* shows how the different transcription units are formed in liver and β cell or pituitary cells. Panel *B* shows how four variant mRNAs are formed. These are described in detail in the text.

Alternate Promoters in the Glucokinase Gene

The hepatic and β-cell GK cDNA sequences diverge from each other at a point that coincides with the splice junction between the first and second exons in the hepatic transcription unit. This observation suggested that different first exons are used in the liver and β-cell transcription units (64, 66). An additional genomic DNA fragment was used to establish this point. An alternate first exon (1β), located upstream from the first exon utilized in liver (1H), encodes the sequence at the 5′ end of the β-cell GK mRNA (67). The cell-specific use of these alternate first exons explains why the N-terminal amino acid

sequence of GK is different in liver and β cells. The hepatic GK transcription unit consists of 10 exons (1H and 2–10) that span a total of 15.5 kb (66). The β-cell transcription unit also has 10 exons (1β and 2–10), but 1β is longer than 1H, and this accounts for the size differences between liver and β-cell mRNA (Figure 2). At least 15 kb separate the 1β and 1H exons; thus the transcription unit of the β-cell gene is at least 30 kb in length. Exons 2–10 are identical in the liver and β-cell transcription units.

The presence of different first exons in the GK transcription units implies that different promoters are used in each cell (see Figure 2*A*). Transcription initiates from a downstream promoter in liver and from an upstream promoter in the pancreatic β cell. A comparison of the sequences immediately upstream from the transcription initiation sites in the liver and β cell did not reveal any similarities, which supports the notion that the gene is regulated differently in these cells. In the liver, transcription initiation occurs within a 4–5 base pair (bp) region located 127-bp upstream from the translation start codon; this appears to be under the control of a TATA box (66). Multiple start sites were mapped ~400-bp upstream from the translation initiation codon in rat insulinoma; these span a 60–65-bp region, and nothing resembling a TATA box is present in this region (67). This use of different promoters is in keeping with the different regulation required to provide the distinct physiologic functions of liver and β-cell GK (64).

Multiple Glucokinase mRNAs

GK mRNAs that are different from the major forms found in liver and β cells have been identified. These mRNAs are the products of specific, alternate splicing events. The first example of a variant GK mRNA was found in rat insulinoma cells (67). This mRNA results from the use of an alternate splice acceptor site in the fourth exon of the gene (example 1, Figure 2*B*). This alternate splicing event removes a 51-nucleotide fragment from the open reading frame, and this results in an in-frame loss of 17 amino acids. This mRNA variant comprises a very small fraction of the total GK mRNA in islets and liver (59), which may explain why the variant was not detected when 15 different rat islet cDNA clones were examined by another group (46). A more complex variant accounts for about 5% of hepatic GK mRNA (42). In this case use of a splice donor site in exon 2 results in the deletion of 52 nt from the reading frame (example 2, Figure 2*B*). In addition, an alternate exon (2A), located between exons 1H and 2 in the gene (see Figure 2*A*), is inserted and this adds 151 nt to the mRNA. The combined insertion and deletion events cause a double frameshift and a net addition of amino acids, giving a protein of 498 amino acids rather than the normal 465. Both of these GK variants have been expressed in prokaryotic cells, and neither is capable of phosphorylating glucose (59, 91).

Two different GK cDNA variants have been isolated from the pituitary gland. One variant (example 3 in Figure 2*B*) results from the use of two alternate splice sites (59). One of these alternate splice sites is in the fourth exon (the one that results in the minor variant shown as example 1). The second is an alternate splice donor site in the second exon that results in the deletion of 25 nt, a frame shift, and premature termination after amino acid 68 (59). A fourth variant, found in pituitary (example 4 in Figure 2*B*), is the same as example 2 except that the 2A cassette exon is not inserted (42, 46). An inactive, 58 amino acid peptide would result from the translation of this variant mRNA. Thus, although the pituitary has GK mRNA (46, 59), the use of alternate RNA splice sites results in mRNAs that, if translated, would encode short peptides that are enzymatically inactive.

In summary, a remarkable diversity of alternate splicing events occurs with this gene product. There are alternate first exons, alternate splice donor and acceptor sites, and a cassette exon is used. Some of these alternate splicing events occur in a cell-specific manner. The regulation of these alternate splicing events needs to be studied further, as this may be an important means of regulating the production of GK in specific cells.

Human Glucokinase

The elucidation of the structure of the rat liver and islet GK cDNAs, and of the rat gene (3, 66, 67), led to the identification and characterization of the human GK cDNAs and gene (56, 81, 107, 112, 113). The deduced amino acid sequences of the rat and human liver GK proteins are 97% identical (3, 112), and the genes have the same general exon/intron structure [Figure 3; and (66, 67, 107, 113)]. Two human liver GK mRNAs, produced by alternate

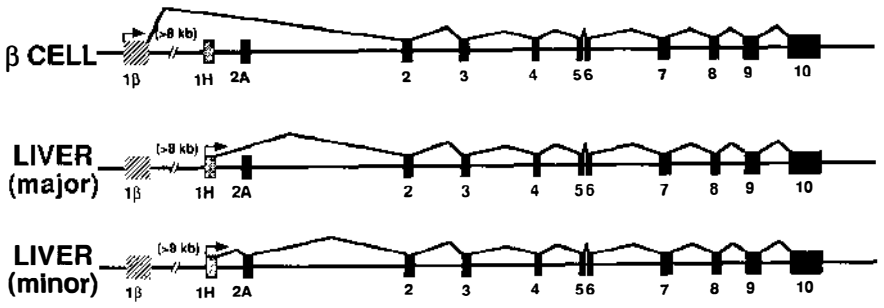

Figure 3 Human glucokinase gene and its transcription units. The overall structure of the human glucokinase gene is similar to that of rat (see Figure 2). Alternate promoters are utilized in the liver and pancreatic β cell. In addition, a cassette exon (2A) is utilized in a minor form of the mRNA found in human liver. The size and location of exon 2A differs from the corresponding exon found in the rat.

RNA processing, involve a 124-bp cassette exon 2A (see Figure 3). The insertion of exon 2A affects the amino-terminal residues of the enzyme and leads to a slightly larger protein of 467 residues. The enzyme produced without exon 2A is 465 residues long. Both are enzymatically active. The human GK gene is located on the short arm of chromosome 7, in band p13 (71, 81). Three microsatellite DNA polymorphisms (long stretches of di-, tri-, and tetra-nucleotide repeat sequences that can vary in length between individuals) are present within or near the gene (71, 81, 113). The identification of these polymorphisms was crucial to the studies that led to the linkage of the GK gene to certain types of noninsulin-dependent diabetes (see below).

Evolution of Glucokinase

The hypothesis that mammalian hexokinases evolved from a common precursor was advanced over twenty years ago (19, 28, 29, 119). According to this view, GK was thought to have evolved from a yeast-like precursor (see Figure 4; model 1). Yeast HK, like GK, has a molecular weight of ~50

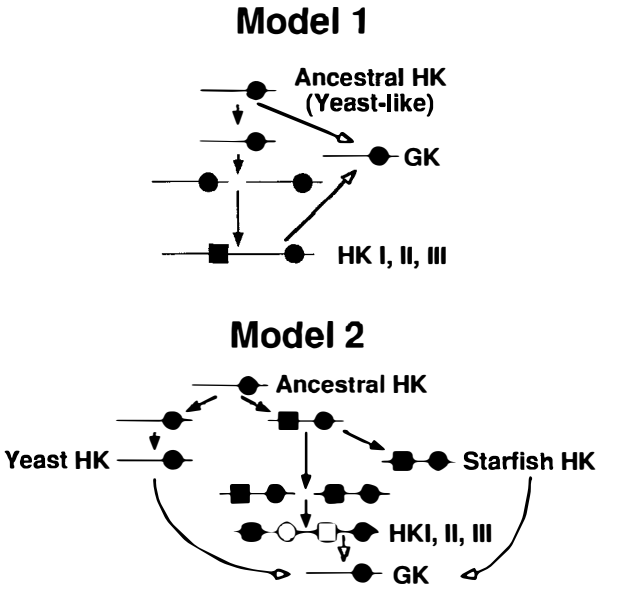

Figure 4 Two models of the evolution of the mammalian hexokinase gene family. In Model 1, a yeast-like HK is presumed to be the precursor, whereas in Model 2 a starfish-like precursor is hypothesized. The symbols are as follows: *solid circle* = the catalytic domain; *solid square* = the G-6-P binding regulatory domain; *open square* = a masked catalytic domain; *open circle* = a masked regulatory domain. The *open arrows* denote the possible routes of GK gene evolution.

kd and it is not inhibited by physiologic concentrations of G-6-P. The mammalian low K_m hexokinases (HK I–III) were thought to have arisen from a yeast-like precursor HK by gene duplication, with tandem ligation of the two products (19, 28, 29, 119). The catalytic site (*solid circle*) in one half of this fusion molecule was presumed to have evolved into a G-6-P binding, regulatory site (*solid square*). According to model 1, the C-terminal half of the HK molecule contains the catalytic site (glucose and ATP binding) and the N-terminal half contains the regulatory site (G-6-P binding). Indeed, the 100-kd HK I binds glucose and ATP, with high affinity, to single sites in the C-terminal half of the molecule, and G-6-P to a single site in the N-terminal half (129).

Representatives of all of the mammalian HKs have been cloned in the past three years. Deduced amino acid sequences obtained as a result of cDNA cloning show that these HKs are indeed closely related members of the same gene family (3, 97–99, 114). Amino acid and coding region nucleotide sequence identity between the family members is 50–70%, and the interspecies sequence identity of a specific family member is at least 85%, and is as high as 97%, as is the case with rat and human GK (see above) (3, 4, 80, 97, 98, 112). Thus there is an extraordinary degree of evolutionary conservation of these genes and proteins. A comparison of the amino acid sequences of the N- and C-terminal halves of HK I with each other, with GK, and with yeast HK, provides support for the gene duplication hypothesis (3, 80, 98).

The availability of cDNA clones led to more direct testing of the location of the functional domains in the 100-kd HKs, and the results obtained from these experiments challenge the hypothesis of HK evolution stated above. For example, the exclusive location of the regulatory site in the N-terminal half of HK I has become debatable (8, 63, 130), since the C-terminal half of HK I, expressed in *Escherichia coli*, has catalytic activity and shows inhibition by hexose 6-phosphates (63). The K_m for ATP, and the K_i for various hexose 6-phosphates of these half molecules, are similar to those of the purified, intact 100-kd enzyme. The amino acids presumed to be involved in the binding of glucose are conserved in both the N-terminal and C-terminal halves of HK I, but site-directed mutations of these residues result in the loss of catalytic activity only when those residing in the C-terminal half are mutated (5, 8). Also, the mutation of one of these residues in the N-terminal half fails to prevent G-6-P inhibition; thus the amino acids thought to be involved in the binding of glucose to the catalytic site in the C-terminal half, although conserved in the N-terminal half, are probably not involved in the binding of G-6-P (8). In view of these observations, it is difficult to imagine how HK I (and other family members) could have simply evolved from a yeast-like HK.

An alternate model has mammalian HKs evolving from a starfish-like HK,

which is a 50-kd enzyme that is inhibited by G-6-P [(130); see model 2 in Figure 4]. Gene duplication and fusion, starting with a starfish-like precursor, would result in a 100-kd protein. According to this model, and in keeping with the glucose, ATP and G-6-P binding data cited above, one would predict a masking of the regulatory site in the C-terminal half (*open square*) and of the catalytic site in the N-terminal half (*open circle*), perhaps as a consequence of the folding of the 100-kd molecule (8, 130). If a starfish-like HK is the precursor of the mammalian HK family, yeast HK may have evolved along a separate path, perhaps from a more primitive precursor. GK still could have evolved from a yeast-like HK; this is unlikely, however, because the exon structures of the GK and HK II genes are very similar. In fact, a comparison of the exon sizes of the GK and HK II genes gives a remarkable clue as to how the latter may have evolved (90). With the exception of exons 1 and 3, the exon sizes in each half of HK II are identical in size to the corresponding exons in GK (90). GK therefore could have arisen from a starfish-like HK as a precursor to the 100-kd HKs, it could have evolved in parallel with the 100-kd HKs, or it could have evolved as a result of a gene splitting from the 100-kd forms, with a decrease in affinity of binding glucose, and a loss of the G-6-P regulatory site. It is apparent that major questions about the evolution of the mammalian hexokinases remain unanswered, but the reagents available should lead to rapid progress in this area.

FUNCTIONAL SIGNIFICANCE OF GLUCOKINASE

The cell-specific regulation of GK discussed later is related to the different functional roles that high K_m glucose phosphorylation catalyzed by GK plays in the molecular physiology of liver and pancreatic β cells. We discuss glucose utilization in the liver and β cell separately.

Role of Glucokinase in the Hepatocyte

The liver plays a central role in maintaining glucose homeostasis [for review, see (35, 43, 87)]. When the plasma glucose is elevated, as occurs after a meal, the liver takes up glucose, replenishes depleted glycogen stores, and then synthesizes fatty acids as another energy storage form. During a fast the liver produces and exports glucose from its glycogen stores, and from gluconeogenic precursors, and thereby provides a continuous energy supply to the brain and red blood cells. These adaptive mechanisms involve several regulatable substrate cycles (35, 87). GK is involved in one of these, the glucose/glucose-6-phosphate cycle. The enzymes that drive these cycles, including GK, are not distributed evenly throughout all hepatocytes, nor are the metabolic processes equally active in all hepatocytes. Instead, there is a "metabolic zonation" in the liver (53).

HEPATIC SUBSTRATE CYCLES Hepatic glucose metabolism involves two major, reversible metabolic pathways. These interrelated pathways control the processes of glycolysis/gluconeogenesis and of glycogen synthesis/degradation. The rate-controlling steps in glycolysis/gluconeogenesis are organized in three cycles through which substrates move in opposing directions under the control of rate-limiting enzymes. These three cycles, named for the substrates involved, are the glucose/glucose-6-phosphate (G/G-6-P) cycle, the fructose-6-phosphate/fructose 1,6-bisphosphate (F-6-P/F-1,6-P_2) cycle, and the phosphoenolpyruvate/pyruvate (PEP/P) cycle [see (35, 87) for detailed reviews]. Within each cycle the product of one enzyme is the substrate of the counteracting enzyme. This would lead to futile cycling were it not for the fact that these cycles are subject to strict regulation by hormonal, neural, and allosteric mechanisms that shift flux in a coordinated manner (35, 87).

The F-6-P/F-1,6-P_2 and PEP/P cycles are most important in controlling the rates of glycolysis and gluconeognesis, as the substrates in these cycles are committed to one or the other of these pathways. The G/G-6-P cycle is involved in glucose entry or exit from the cell and is of additional interest because G-6-P is an uncommitted substrate that can enter the glycogen synthesis, glycolytic, or pentose phosphate shunt pathways. Flux through the G/G-6-P cycle is controlled by the relative activities of glucokinase and glucose-6-phosphatase (G-6-Pase). Hepatic glucose uptake is controlled by the action of GK, and most of glucose output from the liver occurs as a result of the hydrolysis of G-6-P by G-6-Pase (43).

In the basal state, at physiologic concentrations of glucose, the activities of GK and G-6-Pase are nearly balanced, so there is little, if any, net flux through this cycle (17). It has been calculated that each enzyme converts about 0.9 μmol of substrate/min per gram wet weight in liver (43). In isolated hepatocytes, which have lower enzyme activities and rates of flux than liver, net flux may be 0.03 μmol/min per gram wet weight in the direction of G-6-P (indicating a slightly greater activity of GK over G-6-Pase) (17).

As in the other hepatic substrate cycles, there is short-term (seconds to minutes) and long-term (minutes to hours) control of the G/G-6-P cycle. Neither GK nor G-6-Pase are affected by posttranslational modifications (e.g. phosphorylation) that modify the activity of the protein. Acute changes in flux through these enzymes are accomplished by changes in substrate concentration in the case of G-6-Pase and by changes in substrate concentration and the regulatory protein system in the case of GK. The K_m of G-6-Pase for G-6-P is about 2 mM, which is ~10 times greater than the normal intracellular concentration of the substrate, so this reaction proceeds by first-order kinetics under usual circumstances. Thus, when G-6-P levels are high, as in active gluconeogenesis and/or glycogenolysis, flux will be from G-6-P to glucose, and the latter is exported from the hepatocyte. The primary

hormonal signals that result in an acute elevation of G-6-P are an elevated plasma glucagon (cAMP) and a decreased plasma insulin level. These signals, over the long term, result in the induction of G-6-Pase and the repression of GK [see below and Ref. (17)]. The absolute increase of G-6-Pase, coupled with its relative increase over GK, promotes increased flux from G-6-P toward glucose, and export of the latter. For example, a 2-h treatment of isolated hepatocytes with glucagon results in a net flux from G-6-P to glucose of 0.22 μmol/min per gram wet weight. In contrast, the acute elevation of portal vein glucose (or of glucose in an incubation medium) causes glucose to enter the cell through the GLUT 2 transporter and activate GK. This pushes flux in the direction of G-6-P. As long as glycogen synthesis occurs, the G-6-P level is low (as is the level of F-6-P through action of phosphoglucoisomerase) and glucose uptake continues. An increased portal vein glucose also stimulates the release of insulin, and this increases the rate of transcription of the GK gene, which results in increased GK mRNA and GK protein. At the same time, G-6-Pase synthesis is decreased by insulin. The long-term adaptation by insulin therefore involves reciprocal changes in the amount of GK and G-6-P. In isolated hepatocytes such effects result in a net flux toward G-6-P of 0.21 mmol/min per gram wet weight, a 7-fold increase over the basal rate (17).

METABOLIC ZONATION AND GLUCOKINASE Virtually all hepatic functions show zonation, or variation of function in different anatomic regions of the organ (39, 53, 54) For example, gluconeogenesis is most prominent in the periportal area. The gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and G-6-Pase are most abundant in those cells. Glucose uptake for glycogen synthesis, and glycolysis coupled to lipolysis, are most active in the perivenous/pericentral zone. The enzymes pyruvate kinase and GK are found in this zone. The amounts and activities of the enzymes are not constitutive in these zones, nor are the rates of the various metabolic processes. Apparently, these zones are established by gradients of oxygen, substrates and products of metabolic reactions, hormones and other mediators, and by the types and densities of various nerves (39, 53, 54). Of these multiple factors, oxygen and glucagon/insulin gradients are thought to be the most important in establishing the hepatic zonation for carbohydrate metabolism. The periportal to pericentral gradient of oxygen can be about twofold. A 2–4-fold gradient exists for hormones and substrates and can be greater in absorptive and post-absorptive states (53).

The hepatic distribution of GK and GK mRNA has been studied by a variety of techniques, including direct enzyme assays, immunohistochemical localization, and in situ mRNA hybridization. Very low levels of glucokinase

mRNA are detected in the rat liver on neonatal day 1.5, but the pericentral to perivenous gradient is already apparent (77). GK activity is also very low at this time. There is little change until weaning, about postnatal day 15. The response to the ingestion of carbohydrate at this time is instructive. In the fasted animal there is little GK mRNA and no discernable gradient. Four hours after a glucose meal there is a definite pericentral to periportal gradient, and by six hours the mRNA is seen in all cells (77). Changes of GK activity occur more slowly owing to the different turnover times of the protein and mRNA. This experiment indicates that the borders of a zone are not fixed and that they depend upon the metabolic (hormonal) state of the animal. This zonal change is thought to reflect a true induction of GK, presumably by insulin (see below). The nutritional and hormonal conditions that result in the zonal display of GK also cause the disappearance of the periportal zonation of PEPCK. These zonal patterns are thought to reflect metabolic flux. In the example just cited, glucose uptake and glycolysis are increased in the perivenous/pericentral zone as gluconeogenesis is decreased in the periportal zone.

Role of Glucokinase in the Pancreatic β Cell

Pancreatic islet β cells secrete insulin in a glucose-dependent manner. The mechanisms involved have been intensively studied, and several proteins play crucial roles in the process. A key feature underlying the response of the β cell is that the glucose-induced insulin secretory response relies upon changes in the rate of glucose metabolism by these cells (70). Alterations in the rate of glucose utilization are, in turn, tightly linked to changes in the rate of insulin secretion. This coupling is hypothesized to occur as follows. The β cell is electrically active and has a resting membrane potential of about −45 mV [reviewed in (76)]. Increased glucose metabolism results in an increase in the ATP/ADP ratio, which results in decreased conductivity of an adenine nucleotide-sensitive K^+ channel. This leads to depolarization of the β cell (7, 20). The resultant rise in membrane potential opens voltage-dependent calcium channels, thereby allowing the rapid influx of extracellular Ca^{2+} (94, 95) This increase of the intracellular Ca^{2+} concentration is the event that triggers insulin secretion by exocytosis (131). The conductance of adenine nucleotide-sensitive K^+ channels and voltage-sensitive Ca^{2+} channels can be modulated by various hormones and drugs that also affect insulin secretion, but glucose metabolism is required for these effects (76).

The ability of the β cell to respond to glucose involves both glucose transport and phosphorylation, the initial steps in glucose metabolism. Glucose enters the β cell through the high K_m GLUT 2 transporter (83, 115), a process that is not normally rate-limiting, since transport capacity greatly exceeds

metabolic flux (110). Low and high K_m hexokinases exist in the β cell (70, 73), but at physiologic glucose concentrations, GK, with its higher K_m for glucose, accounts for most glycolytic flux (74). Two specific kinetic properties of the enzyme are of paramount importance when the physiologic response of the islet is considered: a K_m that is within the physiologic range of portal vein glucose excursions, and the lack of significant feedback inhibition by G-6-P. The large difference in the capacity for transport versus phosphorylation in the β cell is important in at least two regards. First, the high transport capacity assures a rapid equilibration between extracellular and intracellular glucose. Secondly, the rate of glucose utilization by the β cell is determined by GK.

The rate-controlling effect of GK on glucose utilization by the β cell led Matschinsky and co-workers to suggest that GK is the pancreatic β-cell "glucose sensor" (69, 70, 73). Two recent developments provide additional support for the concept (30, 32, 126). First, mutations of the glucokinase gene are associated with noninsulin-dependent diabetes mellitus (32, 126), as is dicussed below. Second, the constitutive expression of a yeast HK gene in transgenic mice causes increased insulin secretion and hypoglycemia (30). These observations offer new evidence in support of a critical role for GK in determining glucose phosphorylation and insulin secretion rate in the β cell.

CELL-SPECIFIC REGULATION OF GLUCOKINASE

Animal studies provide evidence in support of the differential regulation of GK in hepatocytes and β cells. In animals implanted with insulinomas, the high plasma insulin increases hepatic GK activity and islet cell glucokinase activity is low in the face of a low plasma glucose level (9). The decline of the plasma insulin and the increase of the plasma glucose that occurs upon removal of the insulinoma results in a decrease of hepatic GK activity and an increase in islet GK activity (a V_{max} effect, not to be confused with the K_m effects of the "mnemonic" model discussed above). In other studies a GK cDNA was used to demonstrate that hepatic GK mRNA is decreased in fasted animals and increased in refed animals, but islet GK mRNA is unchanged (50). The differential regulation of glucokinase by insulin in the liver, and by glucose in the β cell, constitutes a potential feedback loop that may be fundamentally involved in the maintenance of euglycemia (9, 67). The structural differences in the transcripts of the hepatocytes and β cell, and the use of alternate promoter/regulatory regions in the gene, provide a structural basis for the differential regulation in these two cell types. The various mechanisms and factors employed to accomplish this differential regulation of GK in the liver and β cell are discussed in detail below.

Regulation of Glucokinase in the Hepatocyte

Nutritional studies provide the foundation for many of the current analyses of hepatic GK gene regulation. GK levels are very low in fasted rats or in animals fed a diet high in fat or protein, and it is difficult to detect the pericentral to periportal gradient of GK expression in such animals. In contrast, rats fed a carbohydrate-rich diet have abundant GK, and the zonal gradient described above is readily visualized. Insulin and glucagon were surmised to be the major regulators of hepatic GK, since the insulin/glucagon ratio is high after a carbohydrate meal and is low after a protein-rich or lipid-rich meal or during fasting. Accordingly, it was expected that insulin would stimulate, and glucagon would repress, GK synthesis. Nutritional studies also helped to define the roles thyroid hormone, glucocorticoids, and biotin have in controlling hepatic GK expression. These effects are discussed in the following sections.

INSULIN AND GLUCAGON The dietary studies cited above suggested that insulin was a major stimulus of GK synthesis. In fact, there is little hepatic GK expression in rats with streptozotocin-induced diabetes, but the administration of insulin returns the enzyme to the normal level (104). It was first shown that these changes in GK activity were accompanied by proportionate changes in the rate of synthesis of the enzyme and that such changes were, in turn, related to changes in the amount of GK mRNA (104). The mechanism of action of insulin on GK gene expression has been studied using both animal models and primary hepatocyte systems. In diabetic rats, in which GK gene transcription is suppressed to a nondetectable level, insulin administration leads to a 20-fold increase in transcription within 45 min (47, 66). It is interesting to note that transcription of the PEPCK gene is reciprocally regulated. As GK gene transcription is increased by insulin, the transcription of the PEPCK gene is shut off (47, 66). Insulin therefore exerts a positive effect on one gene (GK) and a negative effect on another (PEPCK) in the same cell type. The net physiologic result is enhanced hepatic utilization of glucose, since uptake is enhanced and production is decreased. As noted below, glucagon, acting through cAMP, has exactly the opposite effect. This is an example of the coordinate regulation of the enzymes involved in the hepatic substrate cycles, a subject that has been extensively discussed in several recent reviews (35, 43, 87).

The action of insulin on the GK gene appears to be mediated through the insulin receptor. Insulin-like growth factors I and II are effective, but at the high concentrations characteristic of their binding to the insulin receptor rather than to their own receptors (2, 40, 41, 47). The rapid inductive action of insulin on GK gene transcription has led to the suggestion that the effect is

direct and involves preexisting cellular proteins (e.g. transcription factors). Glucose does not affect GK gene transcription in primary hepatocytes, and there is no apparent requirement for glucose in the insulin effect (48). This is in marked contrast to the regulation, by insulin, of genes distal to GK in the glycolytic pathway. Glucose, or a metabolite, is required for the insulin effect on transcription of the bifunctional enzyme and pyruvate kinase genes (87). Possibly, these effects represent an indirect effect of insulin, in that they require glucose metabolism initiated by the primary induction of GK by insulin.

Glucagon decreases the rate of transcription of the GK gene by increasing intracellular levels of cAMP (48). Cyclic AMP has a direct inhibitory effect on GK gene transcription in isolated hepatocytes, and a concentration of insulin that results in a maximal induction of GK gene transcription is overridden by glucagon at a low concentration (6×10^{-10} M). This observation, that an inhibitory effect on transcription is dominant over a stimulatory action, is in keeping with the pattern noted for all enzymes in the three hepatic substrate cycles (35). The inhibitory effect appears to be dominant in all cases studied to date.

In summary, expression of the GK gene requires that glucagon (or other agents that induce hepatic cAMP) levels are low and insulin levels are high. This combination of events occurs after a meal, when dietary carbohydrate needs to be stored and/or utilized by the liver. Thus, nutritional cues are translated into alterations of the rate of transcription of specific genes, and the relative rates of transcription help govern glucose utilization and production.

THYROID HORMONE Thyroid hormones exert a strong effect on lipogenesis through the induction of enzymes that promote the conversion of carbohydrate to triglycerides (45). GK promotes glucose phosphorylation, which leads to an increase in the production of the glycolytic and pentose shunt intermediates that are used in lipogenesis. Because of this chain of events, GK has been considered a lipogenic enzyme. It is therefore not surprising that triiodothyronine (T3), a lipogenic hormone, is also involved in the regulation of the GK gene. Nutrition experiments again provided the framework for subsequent studies of GK gene regulation by T3, and it soon became apparent that T3 and insulin interact in the regulation of the GK gene. One indication of this came from studies in which there was little induction of GK activity in hypothyroid rats that were fasted for 48 h and then were re-fed a high carbohydrate diet (102). In contrast, a very large increase was observed in euthyroid rats treated in the same manner. An analysis of the dietary and hormonal regulation of GK mRNA and GK gene transcription was possible when cDNA probes became available. Basal GK mRNA levels were about

the same in euthyroid and hypothyroid starved rats (44). Hypothyroid rats that were fasted for 48 h and then re-fed a carbohydrate diet for 4 h showed a sluggish and modest increase of GK mRNA. There was no change at 1 h and only a 2-fold increase of GK mRNA at 4 h, but by 24 h this mRNA had returned to the normal value. These changes were accompanied by a 3-fold increase in the rate of GK gene transcription. In contrast, the response in euthyroid rats subjected to the same dietary manipulations was brisk and large. A 14-fold increase in GK gene transcription occurred, with a change noted as early as 30 min. By 1 h there was a 5-fold increase of GK mRNA and by 4 h this had increased 8-fold (44). The inference is that the carbohydrate-induced increase of insulin is the primary effector and that T3 plays an important permissive role (44). Alternatively, it is possible that the effect of glucagon or cAMP (high in fasted animals) is dominant and capable of blocking the effect of T3. The action of T3 is most pronounced in cultured neonatal rat hepatocytes in which it produces an increase in GK gene expression comparable to that seen with insulin alone (79). In these cells the combination of T3 and insulin is greater than the additive responses of T3 and insulin (79). A direct effect of T3 on the GK gene is evident, as GK mRNA is directly increased by T3 in primary hepatocytes isolated from neonatal rat liver.

GLUCOCORTICOIDS Although glucocorticoids and insulin are generally thought to have opposing actions on metabolic processes, both promote glycogen deposition. Glucocorticoids apparently do not have a direct effect on GK gene transcription, but they seem to enhance the response of this gene to insulin. A greater effect of insulin is obtained in the presence of glucocorticoids and is concentration dependent (79). This may be one of the many examples in which glucocorticoids play a permissive role in a metabolic process (36).

BIOTIN GK activity is low in biotin-deficient rats. Insulin normally does not induce GK in such animals, but biotin administration returns the activity of the enzyme to normal (22, 23). Biotin also induces a 3- to 4-fold increase of GK activity in starved rats, and, like insulin, it induces GK precociously in suckling rats (14, 24, 62). Biotin induces an extremely rapid and large increase of GK mRNA in rats fasted for 24 h. Within an hour after addition, GK mRNA increases ~20-fold. This effect is transient, as the GK mRNA quickly declines after 1 h. The kinetics of this response are very unusual, given the fact that GK gene transcription increases by 6–7 fold during this time (14), and the turnover time of GK mRNA is ~30 min (48). Since ~5 turnover times are required for the movement from one steady state to a new one, biotin may alter the turnover time of the mRNA. Some observations suggest that insulin and biotin act by similar mechanisms. For example, the combined

effect of fasting and biotin deficiency on GK expression was not much different than the effect of either alone (62). Also, the combination of glucose, insulin, and biotin treatment of fasted-biotin deficient rats was only slightly more effective than simple biotin treatment (62). However, since the kinetics of the responses to insulin and biotin are very different, these two inducers must be doing something different.

DEVELOPMENTAL REGULATION Hepatic GK is not appreciably expressed in the fetal or neonatal rat. Significant amounts of GK mRNA and protein first appear about day 15, at the suckling-weaning transition (34). Prior to this time, the suckling rat consumes a high fat milk diet that promotes high plasma glucagon and low plasma insulin levels. The key event associated with the initial expression of GK appears to be the decline of glucagon and the increase of insulin that occurs with the consumption of carbohydrates. Studies using primary cultures of hepatocytes isolated from neonatal rat liver before the physiologic onset of GK expression indicate that T3 and glucocorticoids are required in addition to a low glucagon and high insulin level (79). These findings may explain earlier observations which showed that GK could not be induced in very young neonatal rats by insulin alone (128). Plasma glucocorticoids and thyroid hormones begin to increase about 8–10 days after birth, which is about the earliest point that GK expression can be induced, and gradually rise to a peak near the end of weaning. These changes, combined with the diet-induced effects on insulin and glucagon noted above, stimulate the initial expression of GK. The initial (primary) induction of GK expression in neonatal hepatocytes appears to require de novo protein synthesis, possibly of one or more key transcription factors, since there is an 8–12-h lag between the addition of hormones and an increase of GK mRNA (79). The maximal induction occurs at ~24 h under these conditions. In contrast, significant induction can be noted within 4 h, and the maximum level is achieved by 8 h, after a repeated (secondary) stimulation of GK mRNA (79). The chromatin events, transcription factors, and DNA elements involved in the control of GK regulation throughout development are not known.

Regulation of Glucokinase in the Pancreatic β Cell

GLUCOSE Glucose appears to be the major regulator of the expression of pancreatic β-cell GK. Liang et al compared insulin secretion from freshly isolated islets with that from islets maintained in organ culture for 7 days at low and high glucose concentrations (60). Organ cultures of islets in 3 mM glucose showed a 50% reduction in GK activity, whereas those kept in 30 mM glucose showed a 236% increase in GK activity (as compared to GK activity of freshly isolated islets). This 4–5-fold increase was not due to an

indirect induction by insulin released as a result of the high glucose concentration in the medium, since the addition of insulin at 350 ng/ml had no effect on GK activity in islets maintained in medium supplemented with 3 mM glucose. The GK activity in islets cultured at these two extremes of glucose concentration is highly correlated with both glucose-stimulated insulin secretion and glucose usage in the cultured islets, thus further strengthening the concept that GK is a key determinant of this coupled response (61). The mechanism whereby glucose regulates β-cell GK has not been extensively analyzed, but existing studies point to modulation at a posttranscriptional level. In the studies cited above, organ culture for 7 days in either 3 or 30 mM glucose had no effect on GK mRNA, but proportionate 5-fold increases of both GK protein and GK activity were observed (61). Glucose may therefore affect either the translation of GK mRNA or the stability of the protein in the β cell. These studies do not provide support for an effect of glucose on the transcription of the GK gene, through the upstream promoter, but this possibility should be excluded by direct experimentation.

HETEROGENEOUS EXPRESSION Isolated pancreatic β cells differ in their individual responses to glucose (88). For example, insulin secretion, proinsulin content, and membrane potential differ between individual β cells (26, 96). The metabolic responses of β cells differ; some cells have a greater insulin secretory response to glucose than do others. The basis for the metabolic variability of β cells has not been determined, but recently GK was observed to be heterogeneously expressed in β cells (52). While most β cells exhibit low levels of GK immunoreactivity, some β cells stain much more intensely for the enzyme (52). GK appears to be expressed in a random pattern, since no specific gradient of immunostaining, such as that noted in liver, has been observed (52). This observation remains to be fully explored, because it could explain, at least in part, the functional heterogeneity of individual β cells (88). The effect of glucose on β-cell GK heterogeneity has not been studied, but it could be involved in the recruitment of individual β cells into a more metabolically responsive state.

Regulatory Elements in the Glucokinase Gene Promoter

CIS-REGULATORY ELEMENTS IN THE HEPATIC PROMOTER Little is known about the regulatory elements in the hepatic GK gene promoter. A major problem in studying this promoter arises from the fact that there is no permanently established, liver-derived cell line in which the GK gene is expressed or regulated. The lack of this fundamentally important resource has prompted studies in other model systems. For instance, transfection experiments utilizing hepatic promoter fragments containing up to 5.5 kb of 5′

flanking DNA ligated to a reporter gene have been performed in cultured primary hepatocytes (82). Unfortunately no hormonal regulation was observed, although weak reporter gene activity was detected. Detailed studies of the regulatory elements and *trans*- acting factors involved in the hepatocyte-specific expression of the gene, and its multihormonal regulation, await either analysis using transgenic animals or a conceptual or technological advance.

CIS-REGULATORY ELEMENTS IN THE β-CELL /PITUITARY PROMOTER β-Cell-derived cell lines that secrete insulin have been used to define the elements responsible for directing GK gene expression. Shelton et al used both fusion gene and protein-DNA binding experiments to locate two different regulatory elements in the promoter (101). A series of 5′ deletion mutations of the promoter were used to identify a 294-bp segment that was sufficient for efficient transcription of a reporter gene in the HIT M2.2.2 hamster insulinoma cell line. This small region of DNA contains several components that appear to be involved in regulating GK gene transcription. One such element has the sequence TGGTCACCA and is found twice in the 294-bp proximal segment of the upstream GK promoter. This element consists of two inverted repeat segments separated by a single base pair. Mutation of either of these elements, designated Pal-1 or Pal-2, decreases transcription of a reporter gene in insulinoma cells. A second set of sequences, termed the upstream promoter elements (UPE), may also play a role in transcription of the GK gene in the β cell. The three UPEs (UPE-1, -2, and -3) have a similar sequence, CAT(T/C)A(C/G). Mutation of UPE-3 has the most deleterious effect on expression of the reporter gene expression; mutations of UPE-1 or UPE-2 are less detrimental (101). The UPEs bind a nuclear factor that appears to be β-cell specific, but further studies are needed to characterize the factors that bind to the UPE and the Pal elements in the upstream GK promoter, and the role these elements/factors play in β-cell GK gene regulation. Although more progress has been made in analyzing the β-cell promoter than is the case for the hepatic promoter, much remains to be accomplished.

ROLE OF THE GLUCOKINASE GENE IN DIABETES MELLITUS

Undoubtedly, a genetic basis exists for noninsulin-dependent diabetes mellitus (37). Many genes have been considered, and excluded, as candidates [see (37) for review]. The central role of GK in glucose utilization by both the liver and β cell led to the hypothesis that this gene might be involved in diabetes. Meglasson and Matschinsky postulated that small reductions of β-cell GK activity might elevate the glucose set point for insulin secretion,

thus producing a clinical situation in which a higher than normal glucose concentration was required to elicit a given response of insulin secretion (69, 73). This concept was interesting in view of other observations which showed that faulty secretion of insulin is a characteristic pathophysiologic feature of noninsulin dependent diabetes mellitus (NIDDM). If β-cell GK is the glucose sensor for insulin secretion, a defect in the abundance or function of GK could result in an impairment of insulin secretion. The link between certain types of NIDDM, abnormal insulin secretion, and GK gene mutations now appears to have been made.

Froguel et al, using a DNA polymorphism located about 8 kilobases downstream from the 3′ end of the human GK gene (see above), showed linkage between the GK gene and an uncommon type of NIDDM called "maturity onset diabetes of the young," or MODY (32, 71). MODY is inherited as an autosomal dominant trait, and many well-studied pedigrees are available. The first association between the GK gene and MODY was made by analyzing several French pedigrees, and a LOD score of 11.6 was recorded (32). This score indicated that the chance of random association of the GK gene to MODY was less than 1 in $\sim 10^{12}$. In a subsequent analysis of this group of patients, a mutation in exon 7 of the GK gene was detected in one family (126). In short order, several other mutations of the GK gene were detected in other kindreds. In fact, 18 of the 32 MODY families examined have mutations in the GK gene (P. Froguel et al, submitted for publication). At least 16 unique mutations have been identified. These involve missense, nonsense, and frameshift mutations that are scattered through the nine exons of the GK gene that are shared by both the liver and the β-cell enzyme (P. Froguel et al, submitted for publication). The majority are in exons 5, 7, and 8. These mutations have been sorted into two groups. One group appears to affect the active site cleft and/or surface loops that lead into the cleft (exons 7 and 8 are thought to form the surface of the cleft). These mutations cause large reductions of enzyme activity, which is of interest in light of the discussion of enzyme structure presented above. A second group includes residues located far from the active site in a region thought to undergo a conformational change upon glucose binding. These mutations have smaller V_{max} and K_m effects (33). At least one other mutation involves a stop codon at residue Glu^{279}, which results in the production of inactive enzyme.

Although the exact impact of all of these mutations on the function of GK in the patients has not yet been established, at least three kindreds so affected have been shown to have abnormal insulin secretion in response to a glucose challenge (125). This is interesting in view of the fact that one GK allele must be normal in such persons (because it is an autosomal dominant disorder); thus they must have at least 50% of the normal GK activity of unaffected individuals. On the basis of a variety of experiments, Meglasson &

Matschinsky suggested that a 15–30% reduction of β-cell GK activity could increase the threshold of insulin secretion by glucose from 5 mM to 6 mM (73). Whether a similar reduction in the activity of hepatic GK would have an effect of glucose uptake by the liver remains to be established. Linkage with a locus on chromosome 20, near the adenosine deaminase gene, has also been identified in one large MODY kindred (11). Whether this indicates that genes other than GK account for this disease, which is known to present as a variety of different phenotypes (31), or whether the chromosome 20 locus has something to do with regulating expression of the GK gene, or function of the protein (see section above for the discussion of the regulatory protein), remains to be established.

It has not yet been possible to fully assess the association of the GK gene mutations with more typical NIDDM. The belief that this association may be more significant than as a cause of some cases of MODY, which accounts for considerably fewer than 1% of the cases of diabetes in the United States, is supported by the observation of linkage of the GK gene to diabetes in Mauritian Creoles (15), who have a 12% incidence of the disease in adults, and in American blacks (16), who also have a high incidence of the disease. The association of GK with diabetes has received a great deal of attention in the past year. Interested readers are directed to several recent papers (33, 84), and many more can be expected in ensuing years.

CONCLUSIONS

The study of glucokinase is a paradigm of contemporary biomedical investigation. Numerous investigators have made remarkable progress in understanding how this enzyme works, how it evolved, how its activity and expression are regulated, and how it is involved in certain types of diabetes mellitus. This understanding would not have been possible without the interdigitation of information acquired through the disciplines of nutrition, physiology, biochemistry, molecular biology, and human genetics.

A similar, concerted approach is necessary to take the understanding of this critical enzyme to the next level. Every topic discussed in this review is the subject for further study. Such studies will shed light on how gene families evolve, how cell-specific gene expression is accomplished, how *cis/trans* regulation of transcription works, how hormones can stimulate the transcription of one gene in a cell while inhibiting transcription of another, how glucose metabolism is coupled to insulin secretion, how the heterogeneity of gene expression in organs affects their function, and how dietary cues are translated into altered metabolic fluxes. The role of glucokinase in certain forms of adult onset diabetes will be explored for obvious reasons, but mutations that affect the expression of this gene, or the function of its protein product, will provide

information about the nature of these basic mechanisms. The advent of molecular genetics had a major impact on this field, but it is obvious that much remains to be done. As in the past, multiple aspects of glucokinase can be studied by investigators with a spectrum of interests.

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