REVIEW ARTICLE

Mammalian glucokinase and its gene

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INTRODUCTION

Mammalian glucokinase was identified 30 years ago as a distinct form of hexokinase in rat liver. The hexokinases (ATP:hexose 6-phosphotransferases, EC 2.7.1.1) constitute a family of evolutionarily and stucturally related enzymes present in eukaryotic cells from yeast to mammals. In the cells of higher organisms, the physiologically significant substrate for these enzymes is D-glucose. The reaction catalysed by the hexokinases, ATP + D-glucose → ADP + D-glucose 6-phosphate, is the first and obligatory step for glucose utilization after transport of the sugar into the cell. Mammalian tissues contain four different hexokinases which can be isolated by conventional protein separation techniques and for which cDNAs have been cloned. The isoenzymes of the rat have been designated hexokinases type I–IV or A–D in order of increasing negative net charge. The subject of this Review is hexokinase type IV or D, usually called glucokinase.

Glucokinase stands apart from all the other hexokinases by a number of criteria. The first and most striking is its low affinity for glucose. The enzyme is half-saturated with glucose at 6 mM, compared with K_m values in the micromolar range for the three other mammalian hexokinases. This feature led to the discovery of the enzyme and underlies its key role in the physiology of glucose homeostasis. The second hallmark of mammalian glucokinase is its highly typical tissue distribution. The glucokinase gene is transcribed and the mRNA translated into active enzyme only in hepatocytes and insulin-secreting β-cells of the pancreatic islets of Langerhans, reflecting the great functional specialization of this isoenzyme. A third outstanding feature is the developmental and multihormonal regulation of the enzyme, illustrated most dramatically by the transcriptional induction of the glucokinase gene by insulin in the liver.

The distinctive kinetics of glucokinase, its tissue-specific expression and its hormonal regulation were recognized within a few years of the discovery of the enzyme. However, our understanding of these particular characteristics has remained superficial until recently. The main reason for limited progress was the difficulty of purifying the enzyme, hence of raising specific antibodies or obtaining peptide sequence for the isolation of cDNA clones. Once this obstacle was surmounted, studies on glucokinase became a very fertile field of research. The most recent and medically rewarding outcome of this research has been the discovery of mutations of the glucokinase gene as the cause of one subtype of non-insulin-dependent diabetes mellitus (NIDDM). The purpose of this article is to review the recent developments on the structure and function of the glucokinase gene and its gene products, as they relate to our understanding of blood glucose homeostasis. The reader interested in historical perspectives and a complete background on the biochemistry of glucokinase should refer to the classical reviews of Walker [1], Weinhouse [2] and Colowick [3]. Recent commentaries on topical aspects are also available [4–8].

ONE GENE, TWO ENZYMES

The glucokinase gene was first cloned from the rat and the structure of the gene in this species can serve as the standard of reference (Figure 1a). The most remarkable feature is the presence of alternative promoters, responsible for the initiation of transcription at different sites on the DNA in hepatic and endocrine cells. The first clue to the existence of cell-type-specific promoters came from the sequences of two quasi-full-length cDNAs isolated from rat liver and insulinoma libraries [9,10]. The sequences were essentially identical for more than 2000 nucleotides starting from the 3′ ends of the cDNAs, but segments of approximately 100 nucleotides at the 5′ ends were found to differ entirely. The 5′ specific sequences of the cDNAs were mapped to widely separated sites in genomic DNA by Magnuson and co-workers [10,11]. These investigators further identified nine exons, numbered 2–10 in the transcription unit, whose assembly gives rise to the common sequence found in the liver and insulinoma-derived cDNAs. The leader exon encoding the 5′ end of the hepatic mRNA, termed exon 1L, was contained in a phage λ clone which also carried the common exons 2–4. The leader exon for the 5′ end of the insulinoma mRNA, termed exon 1β in reference to the β-cells of the islets of Langerhans, was localized in a different phage clone with non-overlapping genomic DNA. It was therefore concluded that the liver-specific exon 1L was contiguous to the body of the structural gene, whereas the islet-type exon 1β was located at an unspecified distance further upstream. The intervening sequence between the two leader exons has yet to be mapped accurately. Several attempts to isolate rat genomic DNA clones for the entire region have remained unsuccessful in my laboratory, perhaps suggesting unusual features of this DNA. In any event, more than 22 kb of DNA separate the two leader exons in the rat gene (Figure 1a).

The fact that the upstream exon 1β is used exclusively in islet-derived cells, and the downstream exon 1L exclusively in liver, was established by primer extension and nuclease protection experiments and further confirmed by reverse transcription and PCR [10]. It should be noted that the two tissue-specific exons 1 of the glucokinase gene specify not only the 5′ untranslated regions of the islet and liver mRNAs, but also their initial 45 nucleotides of protein coding sequence. It follows that the rat islet and liver glucokinase enzymes will differ in primary structure by 15 amino acids (including initiator Met) at the N-terminal ends of the molecules, for a total sequence of 465 amino acid residues.

In addition to the differential splicing of leader exons associated with the cell-specific control of transcription initiation, other modes of alternative splicing are known to affect glucokinase transcripts. An optional cassette exon has been identified in the rat gene between the originally described exons 1L and 2 (Figure 1a). This cassette exon, termed exon 2A, is retained in a minor fraction of glucokinase mRNA in rat liver [12]. Alternative

Abbreviations used: NIDDM, non-insulin-dependent diabetes mellitus; MODY, maturity-onset diabetes of the young.

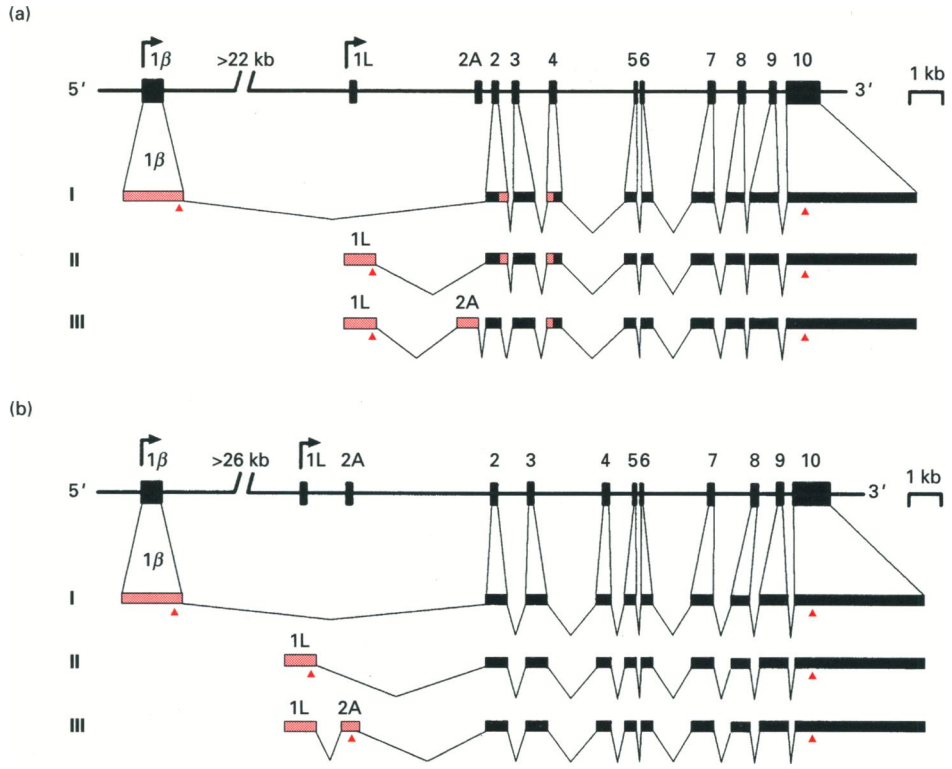

Figure 1 Glucokinase gene in the rat (a) and man (b)

The **top lines** show the exon–intron organization of the genes. Filled boxes represent the exons, curved arrows the sites of transcription initiation. Lines **I–III** show tissue-specific initiation of transcription and alternative splicing. Alternatively spliced exons or parts of exons are coloured red; arrowheads denote the initiation and termination codons. **I**, transcription and pre-mRNA splicing in β-cells of the islets of Langerhans; **II** and **III**, transcription and pre-mRNA splicing in hepatocytes. In the rat, the mRNA in line III is quantitatively minor and encodes an enzymically inactive protein. The relative abundance of mRNAs shown in lines II and III in human liver has not been determined.

donor sites in exon 2 are utilized with low frequency and lead to deletions of either 52 or 25 nucleotides from mature mRNA. Finally, an acceptor site in exon 4 located 51 nucleotides downstream of the major splice site has been described [13]. These different modes of alternative splicing are responsible for the formation of minor mRNA species encoding enzymically inactive polypeptides, as further discussed in the section on the structure of glucokinase.

Recent reports on the cloning of the human glucokinase gene demonstrate that the major structural features of the rat gene have been conserved in the human species [14–16]. Most importantly, the dual promoter arrangement with upstream islet-specific exon 1β and downstream liver-specific exon 1L is very similar in the human and rat transcription units (Figure 1b). As in the rat, the mature glucokinase mRNAs of human islets and liver are assembled from cell-type specific exons 1 and common exons 2–10 [14,15]. The cell-specific exons harbor the initiator ATG triplets, which are located 45 and 48 nucleotides upstream to the donor splice sites in exons 1β and 1L respectively. Reminiscent of the situation in the rat, an alternative transcript in human liver is produced by splicing of an optional cassette exon 2A between exons 1L and 2. In this case, the open reading frame for the protein initiates at an ATG codon situated 42 nucleotides upstream of the cassette exon's donor splice site, predicting a variant N-terminal domain of 14 amino acid residues in the corresponding protein [15]. The gene has been mapped to human chromosome 7p [17].

TISSUE-SPECIFIC EXPRESSION OF THE GLUCOKINASE GENE

The hexokinases taken as a group can be viewed as ubiquitous housekeeping enzymes since glucose phosphorylation is an absolute requirement for further metabolism of this key energetic substrate. Glucokinase, however, subserves highly specialized physiological functions (see below). Accordingly, its gene is expressed with stringent tissue specificity. Early studies using enzymic assays and chromatographic analyses have documented the presence of glucokinase activity only in the liver and islets of Langerhans [18–20] This narrow tissue distribution was confirmed by a sensitive immunoblotting method, which revealed a typical glucokinase polypeptide band with apparent molecular mass of 56 kDa in the cytosolic fraction of liver and islet homogenates, but not in brain, spleen, intestinal mucosa, pancreas, kidney and white adipose tissue [21]. Similarly, by Northern blotting of poly(A)-containing RNA, glucokinase mRNA was initially detected only in the livers and islets of Langerhans of glucose-fed rats [22]. The glucokinase mRNAs of rat liver and islets migrate in electrophoretic gels as 2.5 and 2.8 kb mRNA species respectively. The size difference is explained entirely by the longer 5′ untranslated region of the islet mRNA, encoded by the cell-specific exon 1β. Recently, Hughes et al. [23] have reported the presence of the long version of glucokinase mRNA, reflecting transcription from the β-cell type promoter, in the rat pituitary gland and the corticotroph pituitary cell line AtT-20. However, most if not all of the pituitary mRNA represents

alternatively spliced transcripts which do not code for enzymically active glucokinase[13,23].

As already mentioned, transcription in islet β-cells (and pituitary cells) is initiated exclusively at the upstream promoter and adjacent exon 1β. Conversely, only the downstream promoter and associated exon 1L are active in liver cells. For the rat [10,11] as well as the human [15] genes, the nucleotide sequences of both promoters and flanking regions have been published. Transcription was shown to initiate at well-defined cap sites for the rat and human liver promoters and the human β-cell promoter. In contrast, multiple start sites scattered over a region spanning 62 nucleotides were noted at the β-cell promoter of the rat gene, probably in relation to the absence of a TATA box in this promoter. Elements of DNA sequence with similarity to binding sites for known *trans*-acting factors, cell-specific or ubiquitous, have been highlighted in the promoters and putative regulatory regions of the rat and human genes [10,11,15].

Cis-acting elements important for the transcriptional activity of the β-cell promoter of the rat gene have recently been delineated by Shelton et al. [24]. In short-term transfection experiments, a β-cell promoter fragment, including nucleotides −280 to +14 (with respect to the most downstream of the multiple start sites) was able to drive the expression of the luciferase reporter gene in insulin-producing insulinoma cells of the hamster HIT line, but not in NIH 3T3 fibroblasts. The glucokinase β-cell promoter was indeed more efficient than a rat proinsulin II promoter fragment of comparable length. Block mutations of 10 bp introduced throughout the 280 bp glucokinase promoter led to the identification of two types of *cis*-acting elements of functional significance. One important element is a perfect palindrome TGGTCACCA found at positions −169 to −161 and again at positions −90 to −82. This element was investigated by electrophoretic mobility shift assay for its ability to form specific complexes with nuclear proteins. Several complexes with nuclear proteins from a variety of cell types were observed, suggesting that the palindromic element is a binding site for *trans*-acting factors of the ubiquitous class. A second interesting element, which contributed substantially to promoter activity is found at three locations in the promoter (−215 to −210, −135 to −126 and −102 to −99). The consensus core sequence for this motif, termed the upstream promoter element (UPE) by Shelton et al. [24], is CAT(T/C)A(G/C). Two specific DNA–protein complexes were formed in the electrophoretic mobility shift assay between this type of oligonucleotide and nuclear proteins from β-cell lines. Nuclear extracts from other cell types did not give rise to these complexes, with the possible exception of a pancreatic α-cell line. Interestingly, the consensus UPE motif is identical to *cis*-acting elements of the human proinsulin gene called CT-boxes [25]. In addition, the promoter of the rat proinsulin I gene presents a *cis*-acting element with 5/6 identity with the consensus glucokinase UPE motif [26]. As pointed out by Shelton et al., the β-cell enriched protein factor(s) binding to the glucokinase UPE may be related to or identical with previously described factors which bind to the CT boxes of the human proinsulin promoter or the related element of the rat proinsulin I gene [26,27]. If all these factors prove to be one and the same, they may well represent a key determinant for the transcription of genes typically expressed in β-cells of the islets of Langerhans. It should be pointed out that the pituitary AtT-20 cells, although transcribing the glucokinase gene from the upstream promoter, do not contain the same UPE-binding protein factor(s).

The search for *cis*-acting elements involved in the control of transcription at the hepatic promoter is lagging behind. To my knowledge, liver-specific activity of this promoter in a transient transfection system has not been demonstrated. Hepatoma cell lines in general use do not express the endogenous glucokinase gene [28] and therefore appear an unlikely model for uncovering critical regulatory elements of the promoter. Primary rat hepatocytes can be efficiently transfected by electroporation or lipofection, and high activity of marker genes can be elicited with a variety of viral or cellular promoters [29–31]. However, efforts in our laboratory and elsewhere to express reporter enzymes at the direction of the hepatic glucokinase promoter have remained inconclusive, with plasmid constructs containing as much as 7 kb of 5′ flanking sequence or as little as 110 bp of proximal promoter sequence [31,32]. Inspection of the DNA sequence suggests possible recognition elements for both ubiquitous and liver-enriched *trans*-acting factors. Footprinting by the DNAase I protection assay reveals putative sites of DNA–protein interaction, but their relevance has yet to be tested functionally.

HORMONES AND GENE REGULATION

Alternative promoters allow for versatility in gene control, inasmuch as the initiation of transcription at each promoter in the transcription unit can be regulated by a distinct combination of *trans*-acting factors. Several genes that are expressed at different levels during ontogenic development or in various cell types are known to be transcribed from alternative promoters [33]. The rat glucokinase gene provides an interesting example of transcription unit with tissue-specific promoters which are differentially affected by nutritional and hormonal stimuli.

The level of glucokinase activity in the liver of the rat and other mammalian species has long been known to vary with the nutritional status of the animal. Hepatic glucokinase activity falls during fasting and is restored by glucose refeeding [34,35]. Marked changes in the amount of glucokinase mRNA occur in rat liver under these conditions [36,37]. The message for the enzyme is undetectable by Northern blot analysis in total liver RNA from rats fasted for 24–72 h. Oral glucose administration to such animals causes a rapid, massive and transient accumulation of the hepatic 2.5 kb mRNA for glucokinase. Induction of the mRNA culminates 6–10 h after refeeding, at a level many times as high as seen in the livers of normal animals fed *ad libitum* [36]. In contrast, in the islets of Langerhans, the typical 2.8 kb mRNA for glucokinase is maintained at a constant level during prolonged starvation. Moreover, there is no increase in islet mRNA after an oral glucose load [22]. In line with the mRNA data, Western blotting failed to show any significant change in glucokinase protein amount in pancreatic islets during the fasting–refeeding transition, whereas hepatic glucokinase increased 3-fold within 18 h of glucose refeeding [22]. These results suggest that transcription of the glucokinase gene in liver is turned on by a nutritional signal. On the contrary, the islet β-cell promoter is unresponsive to this stimulus. As a consequence, the enzyme in the islets of Langerhans is expressed constitutively, regardless of the nutritional status of the animal, whereas hepatic glucokinase behaves as a typical adaptive enzyme. This notion has been challenged by Tiedge and Lenzen on the basis of experiments with rats fed a copper-free diet for 3 months [38]. The diet was used to induce atrophy of the exocrine pancreas and a relative enrichment of endocrine tissue, making it possible to assay glucokinase mRNA by Northern blot of poly(A)-containing RNA from total pancreas without prior isolation of islets. In copper-deficient animals, glucose refeeding after a fast caused the rapid appearance of a 2.5 kb mRNA (liver-type) in the pancreas, leading Tiedge and Lenzen to conclude that glucokinase mRNA in islet β-cells was indeed responsive to dietary glucose. In my view, a more likely interpretation is that the glucose-induced 2.5 kb mRNA originated not in islets, but rather

in ectopic foci of hepatocytes that are known to differentiate from putative stem cells in the exocrine pancreas of rodents fed copper-deficient diets, a process called transdifferentiation [39,40].

The response of hepatic glucokinase to glucose refeeding is abolished in animals simultaneously treated with anti-insulin serum [41]. A role for insulin as positive effector of hepatic glucokinase expression in the whole animal is further illustrated in diabetes mellitus. Both enzyme protein and enzyme mRNA are absent from the livers of streptozotocin-diabetic rats. Insulin treatment is accompanied by a prompt build-up of glucokinase mRNA, with a marked overshoot above the normal level for 10 h after the first injection of insulin [42]. At later times of treatment, the mRNA falls towards and below the reference level. The glucokinase protein rises with some lag with respect to the mRNA and reaches normal levels in 16–24 h. The delay in time-course of enzyme accumulation compared to mRNA can be explained by the fairly long half-life of approx. 30 h of glucokinase in rat liver. The mechanism responsible in the first place for the build-up of mRNA and enzyme is a transient burst in the transcriptional activity of the glucokinase gene, as evidenced by run-on assays with isolated liver nuclei [42].

Rat hepatocytes in primary culture have been used extensively to study the role of individual hormones in the regulation of the glucokinase gene. Liver cells isolated from fasted animals and maintained in basal medium are devoid of glucokinase mRNA. Addition of insulin to the medium elicits a time-dependent increase in specific mRNA, with physiological concentrations of hormone. Insulin acts at the transcriptional level, as demonstrated by run-on assays with hepatocyte nuclei [43]. The effect of insulin occurs with or without glucose in the culture medium [43,44]. In this respect, the glucokinase gene stands apart from a group of other genes, such as the L-type pyruvate kinase and S14 genes, which require both insulin and high glucose concentration for transcriptional activation [44–47]. The phorbol ester phorbol myristate acetate, which elicits insulin-like effects in a number of systems [48], does not mimic the effect of insulin on the glucokinase gene (T. Nouspikel and P. B. Iynedjian, unpublished work).

The activation of the glucokinase gene by insulin might be a primary effect, or it might be mediated by an insulin-inducible 'early gene', whose newly synthesized protein product would in turn stimulate glucokinase gene transcription. Insulin induction of glucokinase mRNA was largely or totally suppressed in hepatocytes cultured in the presence of cycloheximide, anisomycin or pactamycin [44,49]. However, a slight stimulation of specific gene transcription was still detectable in these cells by run-on assay. The interpretation of the data was further complicated by a non-specific negative effect of the inhibitors on general transcription [49]. Moreover, protein synthesis inhibitors have been shown to activate protein kinases in cultured cells and to interfere with signal transduction pathways [50,51]. Taken together, the available data suggest that the effect of insulin on the glucokinase gene is at least in part independent of concomitant protein synthesis. Specific activation of the glucokinase gene as early as 30 min after hormone addition (P. B. Iynedjian, unpublished work) supports the idea that the gene is a primary target for insulin action.

Another central aspect of the regulation of hepatic glucokinase is the acute repressor effect of cyclic AMP on the gene. Insulin induction of the message is inhibited by simultaneous addition to the culture medium of glucagon or derivatives of cyclic AMP. At maximal doses of glucagon and insulin, the negative effect of glucagon is dominant and induction is completely abolished [43]. Glucagon or cyclic AMP are also the dominant effectors in cells fully induced by prior incubation with insulin alone. Addition of these effectors in the continued presence of insulin causes an almost immediate cessation of glucokinase gene transcription. Under these circumstances, the mRNA decays with an apparent half-life of 40 min [43]. Interestingly, the negative effect of glucagon is not mimicked by amylin, a polypeptide produced by the pancreatic β-cell and able to counteract the effects of insulin in some target cells [52].

Since cyclic AMP exerts dominant negative control over glucokinase gene transcription, it is legitimate to ask whether insulin might induce the gene by relieving it from basal level cyclic AMP repression. Insulin can antagonize cyclic AMP in liver and adipose tissue by activation of a low-K_m cyclic nucleotide phosphodiesterase termed type III phosphodiesterase [53,54]. At a more distal level in the signal transduction pathway, insulin can oppose cyclic AMP-dependent protein phosphorylation by the stimulation of serine/threonine protein phosphatases [55,56]. We have shown that insulin activation of the glucokinase gene in cultured hepatocytes is prevented by several inhibitors of the cyclic nucleotide phosphodiesterases, in particular by a preferential inhibitor of type III phosphodiesterase [49]. In addition, the inductive effect of insulin was suppressed in presence of low concentrations of okadaic acid, a specific inhibitor of protein phosphatases PP1 and PP2A [49]. These observations underline the importance of the interaction between insulin and the cyclic AMP signalling system for the control of the hepatic glucokinase gene. A highly simplified scheme of the hormonal interactions in the control of specific gene transcription is presented in Figure 2. Glucagon acting via a cyclic AMP-dependent protein kinase is shown to phosphorylate and thereby inactivate a *trans*-acting factor essential for glucokinase gene transcription. Conversely, the putative factor is de-phosphorylated and converted to the active form by an insulin-activated phosphatase. The insulin-dependent kinase cascade resulting in phosphatase activation is derived from the one thought to be active in the control of glycogen synthase activity in muscle ([56–60]; see legend to Figure 2 for details). The putative regulatory factor of transcription is modelled after ADR1, a *trans*-acting factor involved in the control of the alcohol dehydrogenase (ADH II) gene in yeast. In that system, ADR1 is phosphorylated by a cyclic AMP-dependent kinase responsive to glucose and phosphorylation of the factor results in its inactivation and repression of the alcohol dehydrogenase gene [61,62]. The scheme shown in Figure 2 should be regarded as a minimal model. At the gene level, it implies the presence of a single hormone-response element as the target for both the positive effect of insulin and the negative effect of cyclic AMP. It is also possible that insulin and cyclic AMP regulate the gene via separate regulatory proteins and hormone-response elements. Identifying one or several regulatory DNA elements will be the first step to distinguish between these possibilities.

In cultured hepatocytes from newborn rats, the thyroid hormone tri-iodothyronine was shown to be an effective inducer of glucokinase mRNA [64]. The hormone promoted *de novo* appearance of the message, suggesting an effect at the transcriptional level. A direct proof of this point remains to be provided by run-on assays. The effect was additive with that of insulin. In the same study, the synthetic glucocorticoid dexamethasone was devoid of effect by itself, but augmented the response to insulin 2-fold. In the whole animal, the thyroid hormones appear to play a permissive role for the induction of hepatic glucokinase mRNA during the fasting–refeeding transition [65]. It has also been reported that biotin administration to starved rats results in glucokinase gene induction, but this effect may well be indirect and reflect a stimulation of insulin secretion [66].

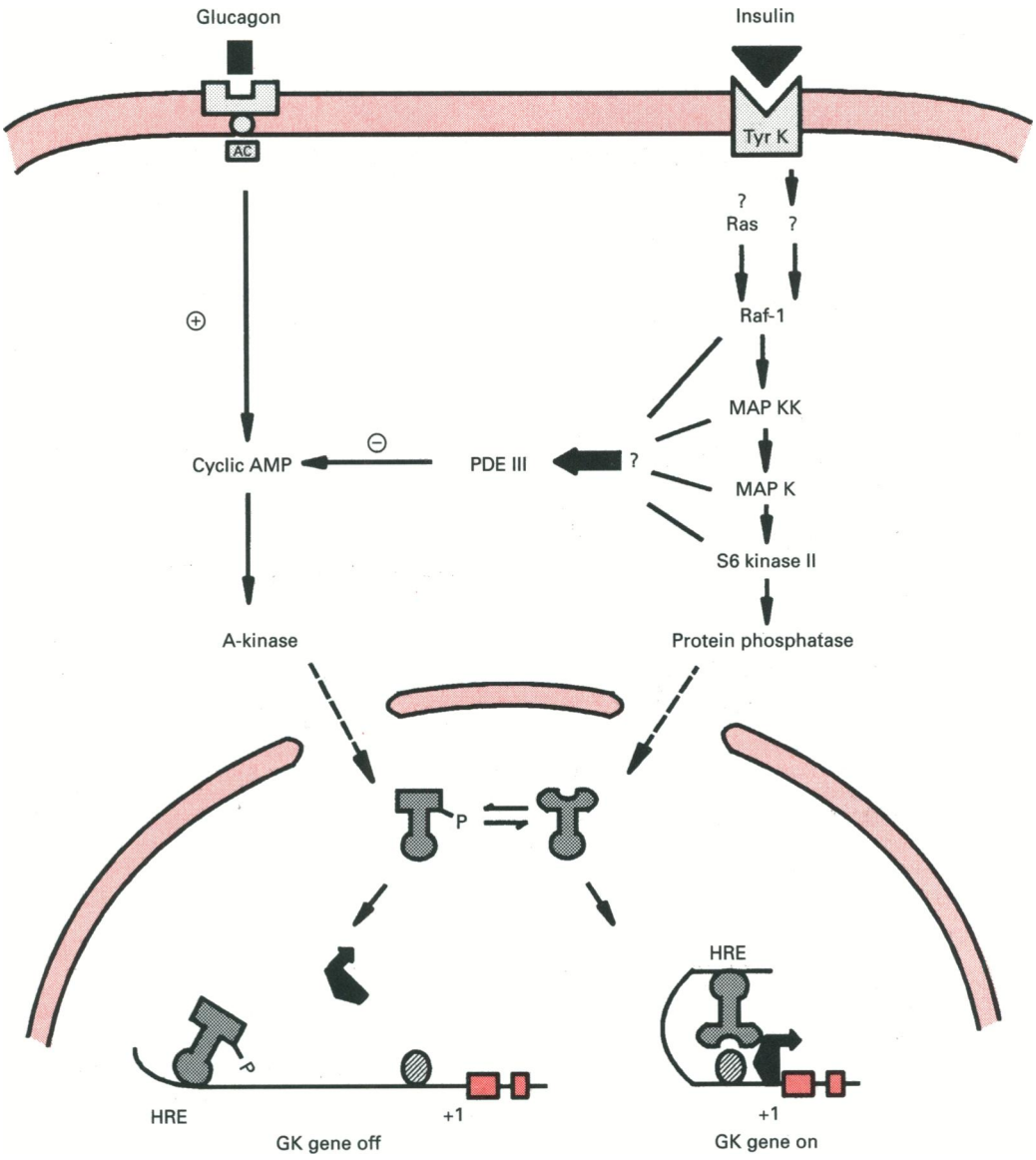

Figure 2 Hypothetical model of glucokinase gene regulation in rat hepatocytes

Two conformations of a putative *trans*-acting factor interconvertible by phosphorylation/dephosphorylation are shown in the cell nucleus. Glucagon binding to its plasma membrane receptor triggers the activation of adenylate cyclase (AC), followed by a stimulation of cyclic AMP-dependent protein kinase (A-kinase) and the migration of the active catalytic subunit of A-kinase to the nucleus. The subunit phosphorylates the *trans*-acting factor, altering the conformation of the transcription activating domain and making it inactive. Insulin activates a protein phosphatase via a protein kinase cascade whose initial steps following the activation of the receptor tyrosine kinase (Tyr K) may or may not involve the cellular proto-oncogenes Ras [60] and Raf-1. The serine/threonine protein kinase Raf-1 phosphorylates and activates a kinase (MAP KK) [59], which in turn phosphorylates and activates a mitogen-activated protein kinase (MAP K, also termed extracellular signal regulated kinase, ERK) [58]. MAP K phosphorylates and activates an insulin-sensitive protein kinase (S6 kinase II) [57] which can specifically phosphorylate a subunit of a protein phosphatase and activate it [56]. The activated phosphatase is shown to migrate to the nucleus and dephosphorylate the *trans*-acting factor, making it competent for the activation of transcription initiation by RNA polymerase II (solid symbol with arrow) at the liver promoter of the glucokinase (GK) gene. Insulin activation of the protein kinase cascade is also shown to result in phosphorylation and activation of a hormone-sensitive cyclic nucleotide phosphodiesterase (PDE III) [54], increasing the turnover of cyclic AMP. The particular kinase for this effect is unknown. The scheme should be taken as a minimal model. The *trans*-acting factor is shown to be bound to the DNA hormone-response element (HRE) even when inactive. It is also possible that the binding affinity of the putative factor is reduced by phosphorylation. Phosphorylation may affect more than one factor, or regulatory subunit(s) of factor(s). Finally, phosphorylation/dephosphorylation of the regulatory factor(s) may take place in the cytoplasm with subsequent migration of the protein to the nucleus (for a review on regulation of transcription by protein phosphorylation, see [63]).

Glucokinase gene transcription in islet β-cells does not appear to be regulated by hormones or other effectors. Down-regulation of islet glucokinase mRNA after prolonged exercise training has been reported, but the decrease in enzyme mRNA was accompanied by a similar decrease in total RNA content of the islets [67]. In RIN insulinoma cells, long-term culture with dexamethasone resulted in a modest increase of glucokinase mRNA relative to γ-actin mRNA. No evidence for a transcriptional effect of the glucocorticoid was produced [68]. In islets of Langerhans maintained in organ culture, glucokinase activity and to a lesser extent glucokinase protein are increased by incubation in the presence of high glucose. However, the level of glucokinase mRNA was unchanged under these conditions [69,70]. The effect on the enzyme concentration might therefore

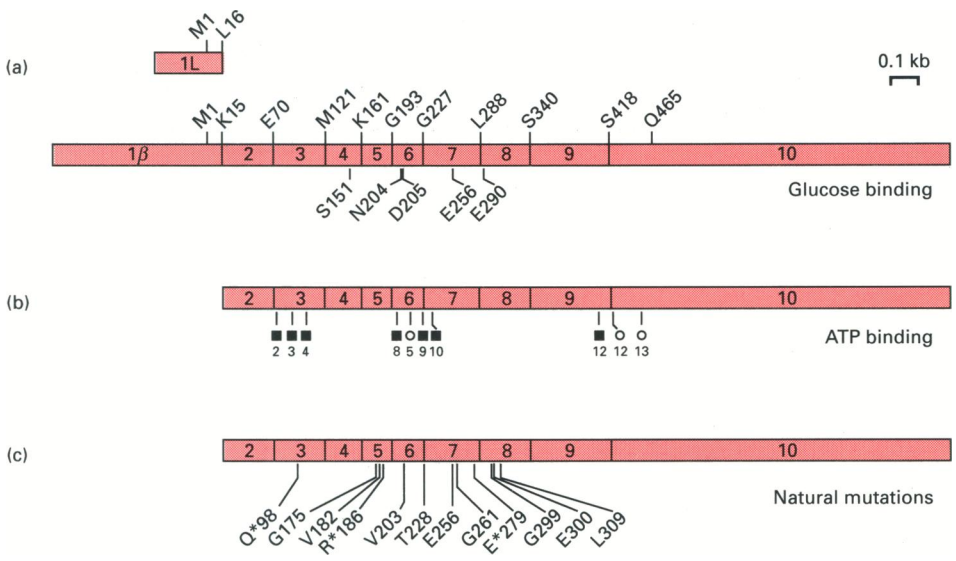

Figure 3 Functional assignments of amino acid residues in glucokinase

The exon structure of islet (1β) and liver (1L) glucokinase mRNAs is represented by the shaded bars. (**a**) **Residues involved in glucose binding.** Above the bar, the first and last amino acids of the enzyme sequence are shown, as well as the last amino acids encoded by each of the exons in the human mRNAs. The one-letter code of abbreviations for amino acids is used. Below the bar, the positions of residues thought to participate in the formation of hydrogen bonds with the hydroxyls of glucose are shown. (**b**) **Sequence elements lining the ATP-binding pocket**: (■) β-strand, (○) α-helix. Thirteen β-strands (numbered 1–13) and 13 α-helices (1–13) have been tentatively assigned along the glucokinase sequence by analogy with the structure of yeast hexokinase [79]. Numbers below the symbols correspond to the numbering in [79]. Residues directly involved in binding ATP are: β-phosphate of ATP, D78–T82 at the loop between β-strands 2 and 3; Mg of Mg-ATP, D205; γ-phosphate of ATP, T228 at the loop between β-strands 9 and 10; adenine of ATP, D409–Y413 between β-strand 12 and α-helix 12. (**c**) **Mutations linked to early-onset non-insulin-dependent diabetes mellitus (MODY).** Residues of the normal sequence affected by nonsense (*) or missense mutations are shown. The amino acid substitutions are given below. For mutations that have been analysed by *in vitro* mutagenesis and bacterial expression, figures in parentheses indicate the -fold decrease in $V_{max.}$ and the -fold increase (+) or decrease (−) in apparent K_m for glucose: G175R (2; +5), V182M (2; +9), V203A (200; +12), T228M (200; 0), E256K (400; −3), G261R (200; −3), E279Q (2; +5), G299R (300; −3), E300K (3; +3), E300Q (0; +3), L309P (100; −4). Data were compiled from [79].

reflect a stimulation of enzyme synthesis at the translational level and/or a stabilization of the enzyme protein.

ENZYME STRUCTURE

The amino acid sequence of rat liver glucokinase has been deduced from the nucleotide sequence of a full-length cDNA by Andreone et al. [9] and independently confirmed by Hayzer and Iynedjian [12]. The sequence is 465 residues in length, with a calculated molecular mass of 51919 daltons and a isoelectric point of 4.85. The sequence is highly conserved in man, with 98% identical residues in the human and rat enzymes [14]. There is high sequence similarity between glucokinase and the three other mammalian hexokinases. The rat hexokinases I–III have amino acid chain lengths of 918, 917 and 924 residues respectively [71–74]. Their genes arose in evolution by a duplication and fusion process from an ancestral gene encoding a primordial enzyme with molecular mass of 50 kDa, similar to the present day yeast hexokinases A and B and mammalian glucokinase [75]. As a reflection of the duplication event, the large-size hexokinases present homologous N-terminal and C-terminal halves, with approximately 50% sequence identity between them [76]. Each half-molecule bears strong similarity to glucokinase. The glucokinase sequence is 49% identical to the C-terminal half of hexokinase I and displays conservative replacements at an additional 15% of the residues; identity with the N-terminal half of the hexokinase is 46% and conservative substitution occurs at 18% of positions [74].

Rat liver glucokinase exhibits 28% identity with yeast hexokinase A and conservative amino acid substitution at 14% of the residues [77]. Extensive similarity in primary structure sugests the conservation of secondary and tertiary structures as well. The crystallographic studies of yeast hexokinase constitute therefore a valuable resource for modelling the mammalian hexokinases and predicting the location of functionally important amino acid residues [78]. In glucokinase, residues directly involved in the binding of glucose, by the formation of hydrogen bonds with the hexose hydroxyls, are thought to be Ser-151, Asn-204, Asp-205, Glu-256 and Glu-290 [9,79]. The positions of these residues in relation to the exon organization of the human glucokinase mRNA are shown in Figure 3(a). Tests of these assignments by site-directed mutagenesis of cDNA and bacterial expression have been initiated by Pilkis and collaborators [80]. The replacement of Asp-205 by Ala has been shown to result in a 500-fold reduction of the enzyme specific activity, without significant change in affinity for either glucose or ATP. This observation is consistent with the putative role of Asp-205 as a base catalyst, promoting the nucleophilic attack of the 6-hydroxyl group of glucose on the γ-phosphate of ATP [80].

A previously unsuspected structural similarity between the hexokinase family and a larger group of ATP-binding proteins has recently been uncovered [81]. Besides the hexokinases, the newly defined superfamily includes a number of bacterial sugar kinases, as well as actin and the heat shock protein Hsp70 and cognate proteins. The three-dimensional structure typical of all these proteins comprises two domains enclosing a large cleft at the bottom of which ATP binds [82]. The ATP-binding pocket is formed by elements of secondary structure which appear to be conserved in similar relative positions in all members of the superfamily. These elements include seven β-strands and three α-

helices [81]. Their tentative locations in the human glucokinase sequence are depicted in Figure 3(b). Further details on residues which participate directly in ATP binding are given in the Figure legend. In yeast hexokinase, binding of a glucose molecule to the enzyme leads to the closing about a hinge of the two enzyme domains mentioned above, resulting in the formation of the ATP-binding site [78]. This mechanism, classically referred to as 'induced fit', is probably also operative in mammalian glucokinase, given the structural similarity between these enzymes.

The liver and islet β-cell forms of glucokinase differ by short N-terminal chains of 14–16 amino acids encoded by tissue-specific leader exons, whereas the remaining 450 residues in the polypeptide sequence are common to both molecules (Figure 1). Is there any particular role for the distinct N-terminal domains ? Earlier biochemical studies have shown that the kinetic properties of glucokinase partially purified from rat liver and insulinoma cells are indistinguishable [83]. More recently, forced expression of cDNAs encoding both types of sequences in transfected NIH 3T3 cells produced glucokinase with similar affinity for glucose in both cases, although there was a suggestion for higher specific enzyme activity in cells transfected with the islet-type cDNA [13]. In yeast hexokinase, the first 20 amino acids at the N-terminus appear to form a flexible chain without any specific assigned function [72]. However, a surprising finding in the glucokinase cDNA expression study with NIH 3T3 cells was that a protein with an incomplete islet-type N-terminal domain, due to deletion of the first seven amino acids, was enzymically inactive [13]. Thus, the function of the tissue-specific N-terminal domains of mammalian glucokinase remains elusive. It has been speculated that this domain might be engaged in protein–protein interaction, perhaps with the glucose transporter GLUT 2, but there is no experimental support for this hypothesis [84].

Some alternatively spliced forms of glucokinase mRNA code for proteins with insertion or deletion of polypeptide segments. The implication of these structural alterations for enzymic activity has been tested by expression of cDNAs in bacteria. One cDNA with insertion of the 151-nucleotide cassette exon 2A and deletion of 52 nucleotides at the end of exon 2 has been isolated from a rat liver cDNA library (Figure 1a, line III). The corresponding message was shown to represent a small proportion of glucokinase mRNA in the livers of glucose-refed rats [12]. At the protein level, a novel domain of 87 amino acids, encoded by exon 2A and the retained part of exon 2, is inserted between Leu-15 and Glu-70 of the conventional glucokinase sequence, in place of the 54 amino acids normally encoded by exon 2. In bacteria, the above cDNA directed the synthesis of a protein with the predicted slightly larger size than authentic glucokinase, but entirely devoid of enzyme activity [85]. Another variant cDNA with deletion of 51 nucleotides at the junction of exons 3 and 4, due to the use of an alternative acceptor site in exon 4, was originally cloned from a rat insulinoma library [10]. Polymerase chain reaction with first-strand cDNA has subsequently shown that mRNAs with the 51-nucleotide deletion were present as minor forms in both islets of Langerhans and liver. The insertion of this cDNA in a vector for bacterial expression of a glutathione S-transferase–glucokinase fusion protein resulted in the synthesis of a hybrid protein without any detectable glucokinase activity, whereas the non-deleted cDNA form produced an active glucokinase fusion protein [13]. Were pre-mRNA splicing regulated, it could represent a means for the control of cellular glucokinase activity, by varying the ratio of messages coding for active enzyme or inactive protein. This hypothesis was tested to explain the decrease in glucokinase activity reported in islets of Langerhans cultured in medium with low glucose concentration. However, a semi-quantitative mRNA assay by reverse transcription–PCR did not reveal a glucose-dependent shift in the relative abundance of the two mRNA species [13].

KINETIC PROPERTIES AND SHORT-TERM REGULATION OF ENZYME ACTIVITY

Three enzymological properties distinguish glucokinase from the other mammalian hexokinases: (i) low affinity for glucose; (ii) lack of inhibition by glucose 6-phosphate and (iii) kinetic co-operativity with glucose. Low affinity for glucose is the diagnostic feature of glucokinase. Half-saturation of the enzyme at 6 mM glucose is put in perspective by considering the K_m of 50, 150 and 7 μM respectively for hexokinases I–III [86]. The physiological advantage derived from half-saturation with glucose at the normal blood glucose concentration is discussed in a separate section. The lack of inhibition by glucose 6-phosphate at low concentration is also unique to glucokinase and relates to the absence of an allosteric binding site for the reaction product [87,88]. Biochemical studies with rat brain hexokinase I have suggested that the glucose 6-phosphate binding site had evolved after gene duplication from the original catalytic site in the N-terminal half of the bipartite enzyme molecule, but this view has recently been shown to be incorrect [89,90].

The third property of glucokinase, its co-operative kinetics with glucose, is of both physiological and theoretical interest. The sigmoid curve of saturation with glucose allows for sharper changes of the reaction rate in response to shifts in the glucose concentration below or above the half-saturation value [91–93]. Glucokinase has a single glucose-binding site and functions in the monomeric state [94]. The classical models of co-operativity for multimeric enzymes are therefore not applicable. A mechanism called 'ligand-induced slow transition' is currently the favoured explanation for the co-operative behaviour of glucokinase [95,96]. The basic tenets of the model are the existence of two kinetically distinct conformational states of the enzyme, and the possibility of slow interconversion between them in function of the ambient substrate concentration. The slowness of the conformational transition relative to the catalytic rate confers to the enzyme a 'memory' of its interaction with the substrate. This property is also the central aspect of the 'mnemonical' model of co-operativity for monomeric enzymes [97,98]. In the latter model, the conformational transition is possible only for the free enzyme, whereas in the ligand-induced slow transition model, the interconversion is also possible for the enzyme–substrate and enzyme–product reaction intermediaries [99]. Kinetic and physicochemical evidence for glucose-induced interconversion between two conformations of rat liver glucokinase has recently been reported by Neet and co-workers. A lag in the reaction velocity could be observed during assay of the enzyme in presence of glycerol, if the enzyme was previously stored with glucose at lower than the assay concentration; conversely, a 'burst' transient was observed when storage of the enzyme was at a higher glucose concentration than during the assay [100]. The physicochemical assay for conformational change relied on spectroscopic measurement of the intrinsic tryptophan fluorescence of glucokinase. A slow enhancement of fluorescence was recorded upon glucose addition and, subsequently, the decay of fluorescence could be followed upon glucose dilution [101]. Given the assumed similarity in tertiary structure between glucokinase and yeast hexokinase, one can speculate that the glucose-induced slow conformational transition of glucokinase is mechanistically related to the classical induced fit occurring in yeast hexokinase upon glucose binding ([78], and see above).

Glucokinase is inhibited by long-chain fatty acyl-CoAs [102].

The inhibitory effect of these compounds is immediate, instantly reversible and occurs with concentrations of fatty acyl-CoAs lower than the critical micelle concentration [103]. On this basis, Tippet and Neet [103] have argued that the inhibition reflects specific binding of the fatty acid derivatives to the enzyme, rather than a trivial detergent effect or unspecific lipid-protein interaction. Kinetic studies have suggested that palmitoyl-CoA and oleoyl-CoA bind to an allosteric site on the glucokinase molecule and elicit a structural change in the enzyme which decreases the binding affinity for glucose and Mg-ATP without impairing $V_{max.}$. Inhibition constants were calculated to be 1.3 μM and 0.75 μM for palmitoyl-CoA and oleoyl-CoA, which appears to be within the range of intrahepatic concentrations [104]. Inhibition of glucokinase activity by fatty acyl-CoAs may be significant *in vivo* in situations of increased lipolysis such as fasting or diabetes mellitus.

Highly purified glucokinase from rat liver can serve as a substrate for cyclic AMP-dependent protein kinase *in vitro* [105]. Phosphorylation was shown to be exclusively on serine and amounted to 1 mol of phosphate/mol of enzyme after prolonged incubation with the kinase. Under these conditions, the glucose affinity and $V_{max.}$ of glucokinase were decreased. The minimal motif Arg-Xaa-Ser, which can serve as substrate site for *in vitro* phosphorylation by cyclic AMP-dependent protein kinase, occurs twice at positions 358–360 and 394–396 in the amino acid sequence of rat liver glucokinase. However, neither of the target motifs Arg-Arg-Xaa-Ser or Arg-Arg-Ser found in proteins phosphorylated *in vivo* by the kinase [106] are present in glucokinase. Indeed, before a prediction based on sequence analysis could be made, attempts to demonstrate a charge shift in hepatic glucokinase according to the nutritional or hormonal condition of the animal had been unsuccessful (P. B. Iynedjian, unpublished work). The possibility of physiological control of glucokinase activity by phosphorylation–dephosphorylation appears therefore unlikely.

An interesting mechanism for the short-term control of glucokinase activity has recently been described by Van Schaftingen and collaborators [107]. These investigators have identified a novel regulatory protein capable of binding to and inhibiting glucokinase in presence of fructose 6-phosphate. Inhibition is relieved in presence of fructose 1-phosphate. The regulatory protein has been purified to near homogeneity. It has a molecular mass of 62 kDa. The formation of a one-to-one complex between this protein and glucokinase in the presence of fructose 6-phosphate has been demonstrated by sedimentation in sucrose gradients. The assembly of the complex was prevented by excess fructose 1-phosphate [108]. A binding assay based on protein precipitation by poly(ethylene glycol) provided direct evidence for the binding to the purified regulatory protein of sorbitol 6-phosphate, an analogue of fructose 6-phosphate, and of fructose 1-phosphate [109]. The model deduced from these studies suggests the existence in rat liver (and in lower amounts in islets of Langerhans [110]) of a glucokinase regulatory protein capable of binding fructose 6-phosphate or fructose 1-phosphate in a reversible and mutually exclusive manner. The fructose 6-phosphate bound form makes contact with glucokinase and inhibits it competitively with respect to glucose. In contrast, the unliganded or fructose 1-phosphate bound form of the regulatory protein does not associate with glucokinase and consequently does not interfere with its activity. These new observations may provide an explanation for the stimulatory effect of fructose on glucose phosphorylation in intact hepatocytes or in the liver of the anaesthetized animal [111,112]. They may also have important implications in normal physiology. In the post-absorptive state, the intra-hepatic concentration of fructose 6-phosphate appears sufficient for substantial inhibition of glucokinase by the regulatory protein. Following a meal, ingested fructose will cause a rapid rise in the hepatic level of fructose 1-phosphate (via fructokinase), with concomitant de-inhibition of glucokinase and thereby increase in the rate of glucose phosphorylation and disposal.

ASSAY AND PURIFICATION OF GLUCOKINASE

The standard method for measuring glucokinase activity in tissue extracts is to assay the rate of formation of the reaction product glucose-6-phosphate with the help of an accessory or 'coupling' enzyme, glucose-6-phosphate dehydrogenase [18,113–115]. The coupling enzyme of choice is the glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*, which is commercially available. This bacterial enzyme uses NAD^+ as coenzyme, the reduction of which is followed spectrophotometrically or, for enhanced sensitivity, fluorometrically. The assay is run in duplicate tubes with 100 mM and 0.5 mM glucose, to score total hexokinase activity and the activity of the three low-K_m hexokinases respectively. The difference between total and low-K_m activity is taken as a measure of glucokinase activity. The above procedure is well suited to measurements in rat liver extracts, in which glucokinase represents 90% of the total hexokinase activity. However, in tissues or tumour cell lines that are rich in low-K_m hexokinases, such as insulinoma cells, the difference in reaction rate at the two glucose concentrations can be small and become experimentally uncertain. Several types of radiometric assays using isotopically labelled glucose have been designed to circumvent this problem [116,117]. In one variant, the synthesis of radioactive glucose 6-phosphate is measured directly by binding the product onto DEAE-cellulose filter disks. The reaction can be performed at high glucose concentration, in the presence of added unlabelled glucose 6-phosphate to inhibit the activity of the low-K_m hexokinases, so as to register only glucokinase activity [116]. Whatever the assay procedure, care should be taken to avoid inactivation of glucokinase *in vitro* (see [118]).

Glucokinase is a low abundance cytosolic protein in liver and is present at even lower levels in islets of Langerhans. From the enrichment factor during purification and quantitative immunoblotting data, it can be estimated that the enzyme represents 0.01–0.1% of total cytosolic protein in rat liver and approximately one-tenth to one-twentieth of this amount in islets of Langerhans. Owing to its scarcity and instability *in vitro*, glucokinase is difficult to purify. An efficient purification scheme has been designed by Holroyde et al. [119], relying essentially on affinity chromatography on Sepharose-*N*-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose. This procedure has been instrumental in allowing Seitz and co-workers to purify rat liver glucokinase to homogeneity and raise specific antibodies to the enzyme in sheep. These antibodies were used initially for the quantification of hepatic glucokinase mRNA by translational assay [37] and subsequently for the immunological screening of a rat liver cDNA library which led to the isolation of the first glucokinase cDNA clone [36].

GLUCOKINASE AND INTEGRATION OF GLUCOSE METABOLISM

The liver can alternatively take up glucose from the blood for the synthesis of glycogen and fatty acids, or release glucose formed via glycogenolysis and gluconeogenesis into the circulation. Net glucose uptake occurs in the postprandial state, when the portal plasma glucose concentration rises above 8 mM, whereas net glucose output occurs at lower glucose levels, namely in the

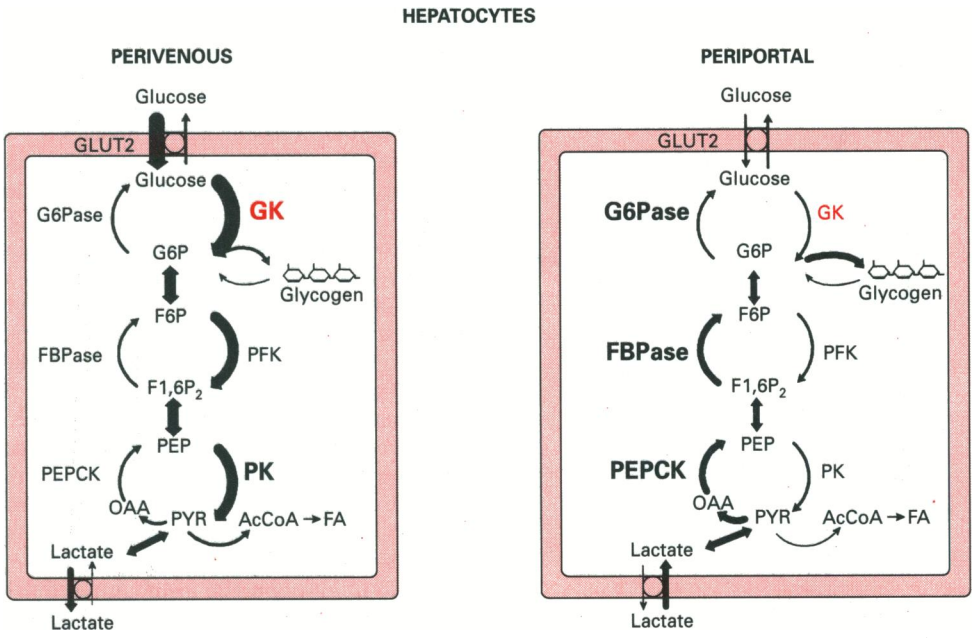

Figure 4 Role of glucokinase in hepatic glucose metabolism according to the concept of functional zonation

Typical hepatocytes of the perivenous and periportal zones of the liver acinus are illustrated. Enzymes enriched in a particular zone are shown in large-size bold letters. The relative thickness of the arrows indicates the importance of carbon flux in a reaction or series of reactions. The metabolic pathways are illustrated in the postprandial state in which net uptake of glucose occurs in the whole liver. In spite of a high glucose-6-phosphatase amount in the periportal cell, most of the carbon flux is channelled toward glycogen synthesis and no net glucose output occurs. See the text for details. Abbreviations: G6P, glucose 6-phosphate. F6P, fructose 6-phosphate; $F1,6P_2$, fructose 1,6-bisphosphate; PEP, phosphoenolpyruvate; PYR, pyruvate; OAA, oxaloacetate; AcCoA, acetyl-CoA; FA, fatty acids; GK, glucokinase; G6Pase, glucose-6-phosphatase; PFK, 6-phosphofructose-1-kinase; FBPase, fructose-1,6-bisphosphatase; PK, pyruvate kinase; PEPCK, phosphoenolpyruvate carboxykinase; GLUT, glucose transporter.

postabsorptive and fasting states [120,121]. By virtue of its particular affinity for glucose, glucokinase is the molecular device which allows the liver to 'assay' the glucose concentration and to shift between net glucose uptake or release. In contrast to the skeletal muscle cell and adipocyte, the hepatocyte is endowed with a high-capacity glucose transport system which is independent of insulin [122–124]. Transport across the cell membrane occurs by facilitated diffusion via the specific glucose transporter isotype called GLUT 2 [125]. The transporter can move glucose in and out the cells, is half-saturated at around 20 mM glucose and is expressed at high level, such that the transport step is never rate-limiting for glucose metabolism. This system allows free equilibration of glucose inside the cell and glucose in the extracellular fluid. Consequently, fluctuations of the blood glucose concentration are instantly followed by parallel changes in the intracellular glucose concentration. These variations will be monitored by glucokinase in the cytosol of the cell and translated into an increase or decrease in the rate of synthesis of glucose 6-phosphate destined for glycogen synthesis or glycolysis.

A second enzyme directly involved in hepatic glucose uptake or release is glucose-6-phosphatase. In the postabsorptive and fasting states, glycogenolysis and gluconeogenesis provide a steady supply of glucose 6-phosphate. Glucose-6-phosphatase converts this metabolite into free glucose for release into the circulation. Glucose-6-phosphatase and glucokinase catalyse opposing reactions, giving rise to a substrate cycle between glucose and glucose 6-phosphate [126]. The net carbon flux between these substrates, hence the net movement of glucose in or out of the liver, is determined by the difference in rates (if any) of the two reactions. This in turn will depend on the amounts of the two enzymes and on the balance of all factors involved in the acute control of the reactions rates. Hyperglycaemia and dietary fructose (acting via fructose-1-phosphate and release of the glucokinase inhibitory protein) are factors which enhance the rate of the glucokinase reaction and promote glucose uptake in the postprandial period. Conversely, hypoglycaemia causes a decrease in the rate of glucose phosphorylation and favors hepatic glucose output in the fasting state. Unrestrained glucose output will ensue from a reduction of the glucokinase enzyme concentration in prolonged fasting or diabetes, due to the turning off of specific gene transcription.

Although substrate cycling is a valid notion for the liver taken as a whole, it appears to be limited in extent at the single cell level owing to the functional specialization of individual hepatocytes. The unidirectional enzymes of gluconeogenesis and glucose-6-phosphatase are enriched in the periportal hepatocytes of the liver acinus, whereas glucokinase and the unidirectional enzymes of glycolysis predominate in the perivenous hepatocytes. This topological separation of metabolic pathways is referred to as metabolic zonation [127]. Concentration gradients for oxygen, substrates and hormones in the liver microcirculation are thought to be responsible for region-specific gene expression. Both immunohistochemistry and microdissection data have shown that the concentration of glucokinase is approximately two times higher in perivenous than in periportal hepatocytes, and inversely for glucose-6-phosphatase [128–131]. Glucokinase mRNA has been localized to the perivenous areas by *in situ* hybridization [132]. In the perfused rat liver, glycogen synthesis from glucose was shown to take place primarily in the perivenous zone, whereas the periportal cells synthesized glycogen preferentially from lactate and pyruvate [133,134]. Thus, metabolic zonation may account for the capacity of the liver to synthesize glycogen after a glucose load by a direct route from glucose itself as well

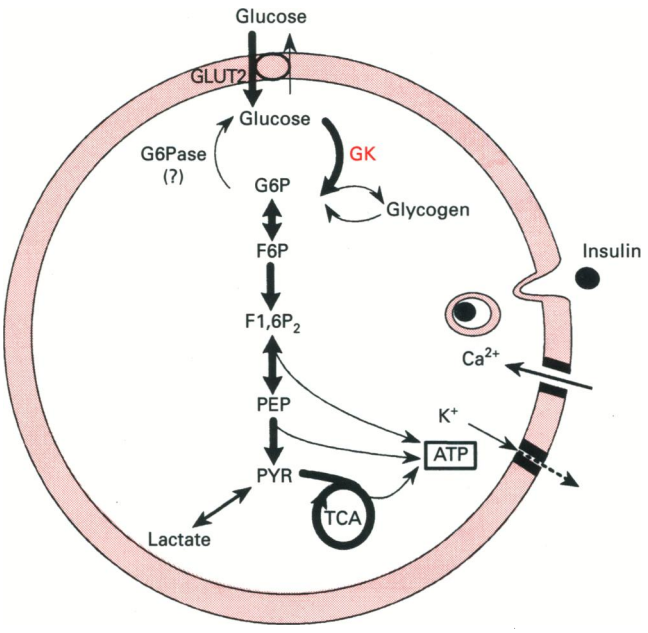

Figure 5 Role of glucokinase in the physiology of insulin secretion in the β-cells of the islets of Langerhans

Glucose metabolism is necessary for regulated insulin release. The glycolytic pathway and tricarboxylic acid cycle (TCA) are schematically illustrated. The metabolic flux is controlled at the glucokinase step. The production of ATP results in a rise in the ATP/ADP ratio, which effects the closure of ATP-sensitive potassium channels. The ensuing membrane depolarization causes the opening of voltage-sensitive calcium channels and an influx of Ca^{2+} ions necessary for insulin release. See the text for details, and the legend to Figure 4 for explanation of abbreviations.

as by an indirect route from lactate [135]. The direct route would be active mostly in perivenous hepatocytes, which would take up glucose from the blood and phosphorylate it via glucokinase. Part of the glucose 6-phosphate would be converted directly into glycogen and the remainder metabolized to lactate by glycolysis. After release into the general circulation, lactate would be taken up by periportal hepatocytes and converted into glycogen via the gluconeogenic pathway. It is noteworthy in this scheme (illustrated in Figure 4) that hepatic glucokinase handles all the glucose eventually utilized for glycogen synthesis, even though some of the carbon flux after glucose phosphorylation is diverted via glycolysis and gluconeogenesis prior to the synthesis of glycogen [136].

The physiological impact of glucokinase gene expression in β-cells of the islets of Langerhans is well understood. The single most important stimulus for insulin secretion by the β-cells is a rise in the blood glucose concentration. Cellular uptake of glucose and metabolism via the glycolytic pathway and tricarboxylic acid cycle are requisites for the insulinotropic effect [137]. Three biochemical properties of the β-cell are essential for a physiological insulin secretory response: (i) rapid equilibration of the glucose concentrations in the extra-and intra-cellular compartments; (ii) rate-limiting step of glucose metabolism catalysed by glucokinase and (iii) coupling link between rate of glucose metabolism and the insulin release process.

The glucose transporter isotype normally expressed in islet β-cells is GLUT 2 [125,138,139]. Maximal rates of glucose transport measured in isolated islets or dispersed β-cells exceed the rates of glucose metabolism approximately 100-fold, making it clear that metabolism is not controlled at the plasma membrane transport step [140–142]. Several lines of evidence suggest that the phosphorylation of glucose is the rate-limiting step in whole islets, and by inference in β-cells, which constitute approx. 80% of the islet mass [143]. Enzyme assays under $V_{max.}$ conditions indicate that total hexokinase activity, including both low-K_m hexokinase(s) and glucokinase, is by far lower than the activities of the two regulatory enzymes of glycolysis, 6-phosphofructo-1-kinase and pyruvate kinase [144]. Although glucokinase accounts for only 25% of total hexokinase activity in islet extracts, it appears to contribute approx. 90% of the glucose 6-phosphate synthesized at normal glucose concentration in intact islet cells, owing to effective inhibition of the low-K_m hexokinase(s) by the ambient glucose 6-phosphate concentration [145]. It is therefore glucokinase which assumes control over the carbon flux in the glycolytic pathway, allowing physiological fluctuations of glucose concentration to translate into alterations of the overall glycolytic rate. The coupling link between glycolytic metabolism and insulin secretory activity is thought to reside in ATP-sensitive potassium channels in the β-cell plasma membrane [146–148]. These channels close following a rise in the intracellular ATP/ADP ratio. The ensuing membrane depolarization and electrical activity cause the opening of voltage-gated calcium channel, an influx of calcium ions from the extracellular fluid into the cell and ultimately, via steps that remain to be elucidated in detail, the release of insulin from secretory granules (Figure 5). In the final analysis, the glucose responsiveness of the entire system is vested in glucokinase. This notion is encapsulated in the term 'glucose sensor' used by Meglasson and Matschinsky to designate the β-cell glucokinase [149].

A recent immunohistochemical study by Jetton and Magnuson [150] has suggested large differences in glucokinase amount between individual β-cells of single islets. Whether β-cells enriched in or devoid of glucokinase also vary in the levels of other enzymes is unknown, but it is plausible that metabolic zonation occurs in the β-cell population of the islets. This may represent an underlying factor for the reported functional heterogeneity among these cells [151]. Glucose 6-phosphatase immunoreactive protein and enzyme activity have been detected in whole islet extracts [152]. Moreover, substrate cycling between glucose and glucose 6-phosphate has been demonstrated [153,154]. Whether glucokinase and glucose-6-phosphatase coexist in the same cells and whether glucose cycling plays a role in the regulation of insulin secretion remains to be determined. It is also interesting to note that tumoural β-cell lines can present marked alterations in enzyme expression as compared to the parent cells. An example is provided by an insulin-producing rat insulinoma cell line (RINm5F) studied in our laboratory. The cells have near-normal levels of glucokinase, but dramatically overexpress hexokinase II [83]. We have proposed that the imbalance between glucokinase and low-K_m hexokinase activities in this and probably other β-cell lines is a major determinant for the lack of glucose-stimulated insulin release within the physiological range.

A MOLECULAR DISEASE OF GLUCOKINASE

NIDDM is a common metabolic disorder characterized by impaired insulin secretion in response to glucose and resistance of the target tissues to the action of insulin, including exaggerated hepatic glucose output in the postabsorptive state. Both defects contribute to the development of fasting hyperglycaemia at full-blown stages of the disease. The aetiology of NIDDM is not understood in detail, but is known to involve both genetic and environmental factors [155]. As a general rule, the disease is classified among the multifactorial–polygenic syndromes. How-

ever, there is a rare and mild form of NIDDM with simple autosomal dominant mode of inheritance, the early-onset NIDDM or maturity-onset diabetes of the young (MODY) [156]. The clear-cut inheritance of MODY makes this syndrome attractive for the search of disease genes linked to NIDDM [157]. Candidate genes can be targeted for study, based on our extensive biochemical knowledge of glucoregulatory mechanisms. Because of the major impact of glucokinase on blood glucose homeostasis, in the control of hepatic glucose disposal as well as in the regulation of pancreatic insulin secretion, the glucokinase gene has been placed high on the list of potential diabetes susceptibility genes.

Two separate studies have recently reported linkage of the MODY phenotype to the glucokinase locus in a number of multigenerational pedigrees with the disease [158,159]. The transmission of glucokinase alleles was followed using two polymorphic microsatellite DNA markers in the immediate vicinity of the gene [17,158]. Specific mutations have been identified in MODY patients in several of the above families [160,161]. The mutations were not found in unaffected relatives nor in control subjects in the general population, suggesting their critical role in the disease. The reported mutations include nonsense, missense and splicing site mutations. The positions of the amino acid residues affected by nonsense or missense mutations are shown in Figure 3(c). In order to assess the effect of the missense mutations on the enzymic activity of glucokinase, Gidh-Jain et al. [79] have reproduced these mutations by *in vitro* mutagenesis of a human islet cDNA and analysed the kinetic properties of the resulting proteins after bacterial expression. The amino acid substitutions were shown to have more or less pronounced deleterious effects on the $V_{max.}$ and/or glucose affinity of the enzyme, as detailed in the legend to Figure 3.

What are the clinical consequences of mutations in the glucokinase gene? Patients from four MODY kindreds with four distinct glucokinase mutations were evaluated for islet β-cell function by Velho et al. [162]. The patients presented a normal first-phase elevation of plasma insulin in response to a priming injection of glucose, but failed to sustain adequate insulin levels during a continuous glucose infusion set to maintain the plasma glucose concentration at 10 mM. In the basal state, these subjects also displayed inappropriately low plasma levels of insulin in relation to the slightly elevated blood glucose levels. Together, data from these patients are compatible with an increase in the glucose threshold for insulin secretion. Such a defect is a predicted consequence of a reduction in β-cell glucokinase activity, as shown in a theoretical model of glucose-stimulated insulin secretion presented several years ago by Meglasson and Matschinsky [149]. Investigations of hepatic glucose metabolism in subjects with MODY have not yet been reported. From our current knowledge, the MODY syndrome associated with mutations of glucokinase emerges as a disorder characterized by mild hyperglycaemia appearing during childhood, resulting primarily from a discrete defect in glucose-induced insulin secretion [161,162]. In that, it differs from more prevalent forms of NIDDM in which the primary abnormality appears to be the resistance of the liver and peripheral tissues to the action of insulin [163]. Mutations of the glucokinase gene could also be a factor in some forms of NIDDM with late onset, although their occurrence in these forms appears to be uncommon [164,165].

CONCLUSIONS AND PROSPECTS

The discovery of glucokinase mutations as a cause or contributing factor of MODY and other forms of NIDDM will come to be regarded as a paradigm in human genetics. Given its essential role in blood glucose homeostasis, glucokinase was near the top of the list of candidate genes potentially involved in NIDDM. Conversely, the strong evidence now produced for the association of glucokinase mutations with NIDDM reinforces the physiological importance of the enzyme. Classically, genetic enzyme deficiencies with clinical consequences are recessive diseases, since a single normal allele can in principle sustain sufficient enzyme synthesis for unimpaired cellular function. Glucokinase, however, is a rate-limiting enzyme of metabolism in liver as well as islet β-cells and it is to be expected that seemingly small deficits in enzyme activity would lead to both exaggerated hepatic glucose output and reduced insulin release at a given blood glucose level. To put things in perspective, it should be reminded that hepatic glucokinase activity is reduced by 'only' 50% in rats after 3 days of fasting, a long fast for rodent species. An intriguing question that remains to be answered is whether mutations in the regulatory regions of the tissue-specific promoters or in the leader exons 1β or 1L occur in human disease, affecting either islet or liver glucokinase separately.

The technique of targeted gene disruption in embryonic stem cells [166] makes it feasible to produce mouse strains with partial (heterozygous) or total (homozygous) glucokinase deficiencies. Such strains would provide invaluable animal models for studying: (i) the metabolic consequences of liver and/or islet glucokinase deficiencies; (ii) the interaction of the genetic background and environmental (e.g. nutritional) factors in the development of glucose intolerance or diabetes and (iii) the effects of drugs and other preventive or therapeutic manoeuvres.

Glucokinase will continue to attract great interest as a model system for studies of differential gene regulation in various cell types. The β-cell specific promoter has already been dissected into discrete binding sites for transcription regulatory *trans*-acting factors. The characterization of these proteins, the cloning of their genes and their comparison with factors involved in the transcription of other β-cell expressed genes (e.g. the insulin gene) will provide important clues on the differentiation of this endocrine cell type. At present, it appears that the β-cell promoter is not acutely regulated by hormones or other signals, but this remains to be definitively established. The possibility of specific regulation of enzyme synthesis at the translational level should also be pursued. Although the distribution of glucokinase is stringently restricted in the organism, it cannot be ruled out that cell-types other than hepatocytes and endocrine β-cells express the enzyme.

Transcription of the glucokinase gene in hepatocytes is absolutely dependent on the presence of insulin, at least in the rat, and indeed the activation of the glucokinase gene has emerged as a major hepatic effect of insulin. It will be important to determine whether this holds true in human hepatocytes. The effect of insulin is long-term in that it will affect the synthesis of a fairly stable enzyme; it is nevertheless a very rapid effect when monitored by transcriptional assay. Just as impressive is the instant turning off of gene transcription under the influence of cyclic AMP. Rapid repression of gene transcription contrasts with the delayed and blunted response at the enzyme level. Stringent and acute control of gene transcription for an enzyme with relatively long half-life seems somewhat paradoxical; its value may reside in the sparing of high energy nucleoside trisphosphates when carbohydrate food becomes scarce. As regards molecular mechanisms, the regulation of the glucokinase gene in the hepatocyte remains much of a mystery. A decisive step would be accomplished if one could understand why the expression of the gene is extinguished in hepatoma cell lines. The developmental regulation of the gene in liver, with the initial appearance of the mRNA at weaning time, may also provide

interesting cues when studied at the molecular level. Finally, working backward from the gene to regulatory *trans*-acting factors and signal transduction cascades is an exciting prospect that is sure to add important new insight into the mechanism of insulin action. In the course of such studies, more may be learned about insulin resistance and non-insulin-dependent diabetes mellitus.

I thank Dr Milena Girotti for designing the figures. Thanks are also expressed to Dr Philippe Froguel for making preprints available before publication. Research in my laboratory was supported by grants from the SNSF and the Wolfermann–Nägeli Foundation.

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