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Hexokinase isoenzymes of RIN-m5F insulinoma cells

Expression of glucokinase gene in insulin-producing cells

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We have analysed the pattern of expression of the hexokinase isoenzyme group in RIN-m5F insulinoma cells. Three hexokinase forms were resolved by DEAE-cellulose chromatography. The most abundant isoenzyme co-eluted with hexokinase type II from rat adipose tissue and displayed a K_m for glucose of 0.15 mM, similar to the adipose-tissue enzyme. Hexokinase type II was in large part associated with a particulate subcellular fraction in RIN-m5F cells. The two other hexokinases separated by ion-exchange chromatography were an enzyme similar to hexokinase type I from brain and glucokinase (or hexokinase type IV). The latter isoenzyme was identified as the liver-type glucokinase by the following properties: (i) co-elution with hepatic glucokinase from DEAE-cellulose and DEAE-Sephadex; (ii) sigmoid saturation kinetics with glucose with half-maximal velocity at 5.6 mM and Hill coefficient (h) of 1.54; (iii) suppression of enzyme activity by antibodies raised against rat liver glucokinase; (iv) apparent M_r of 56500 and pI of 5.6, as shown by immunoblotting after one- and two-dimensional gel electrophoresis; (v) peptide map identical with that of hepatic glucokinase after proteolysis with chymotrypsin and papain. These data indicate that the gene coding for hepatic glucokinase is expressed in RIN-m5F cells, a finding consistent with indirect evidence for the presence of glucokinase in the β-cell of the islet of Langerhans. On the other hand, the overall pattern of hexokinases is distinctly different in RIN-m5F cells and islets of Langerhans, since hexokinase type II appears to be lacking in islets. Alteration in hexokinase expression after tumoral transformation has been reported in other systems.

INTRODUCTION

The hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) isoenzymes catalyse the first step of glucose metabolism in mammalian cells. Four hexokinase types, numbered I to IV in the order of elution from the anion-exchange resin DEAE-cellulose, have been distinguished [1,2]. Although most cell types contain more than one hexokinase type, the expression of the diverse isoenzyme forms is to some extent tissue-specific. Thus, hexokinase type I is prevalent in brain and kidney, hexokinase type II in adipose tissue and striated muscle, and glucokinase (or hexokinase type IV) in liver [2–5]. In addition to tissue-specific factors, dietary and hormonal stimuli are known to be involved in the regulation of glucokinase and hexokinase type II [6,7]. Furthermore, tumoral transformation of several cell types has been associated with an altered pattern of hexokinase expression and intracellular localization as compared with the tissue of origin [8–10]. The mechanisms underlying tissue-specific, hormonal and transformation-dependent effects on hexokinase expression remain to be elucidated.

In this work we have analysed the pattern of hexokinases in the RIN-m5F endocrine cell line. These cells were established in culture from a radiation-induced insulinoma of the rat and exhibit continued production of insulin, a feature characteristic of the β-cell of the islet of Langerhans [11]. Previous work from our laboratory showed that a gene product closely related to or identical with liver glucokinase was expressed in the islet of Langerhans of the rat pancreas [12]. The major question in the present study was whether the expression of the glucokinase gene is retained in RIN-m5F cells.

This issue was raised for the following reasons. First, de-differentiation attending malignant tumour formation in liver is known to be accompanied by the extinction of glucokinase gene expression [8]. Thus glucokinase can serve as a useful marker of cellular differentiation for the hepatocyte and, presumably, for endocrine cells of the pancreas. Second, glucokinase appears to be a rate-limiting enzyme of glycolysis in the β-cell of the islet of Langerhans and, as such, is thought to play a key regulatory role in glucose-induced insulin secretion [13,14]. The presence or absence of glucokinase in the RIN-m5F cell line, the most prevalent tissue-culture model system of insulin-producing cells, has been a matter of debate [15,16]. Therefore we considered it important to seek direct evidence for the presence of the glucokinase gene product in these cells, as well as to define the pattern of expression of the entire hexokinase isoenzyme group.

EXPERIMENTAL

Cell culture

The RIN-m cell line was established in culture by Gazdar *et al.* [11]. Clone 5F was derived by Bhathena *et al.* [17] and has been subcloned in our Institute. Cells

Abbreviation used: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate.

from this subclone have now been maintained in culture for 5 years with stable insulin content [18]. For the present experiments, cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal-calf serum and penicillin/streptomycin at concentrations of 100 units/ml and 100 μg/ml respectively. Cells were harvested at sub-confluence by scraping the culture dishes with a rubber policeman. The cells were then thoroughly washed with phosphate-buffered saline (10 mM-sodium phosphate buffer, pH 7.4, 140 mM-NaCl).

Preparation of cytosol and particulate fraction.

Washed cells were allowed to swell for 10 min at 4 °C in a hypo-osmotic solution containing 20 mM-Tris/HCl, pH 7.0, 5 mM-EDTA, 4 mM-$MgSO_4$, 2 mM-dithiothreitol and 20 mM-glucose (solution A). NaCl was added (final concn. 80 mM) and cells were homogenized in a Teflon glass homogenizer by 30 strokes of a pestle driven at 1500 rev./min. Cytosol and particulate fraction were separated by centrifugation at 149000 $g_{av.}$ for 60 min. After withdrawal of the cytosol, the pellet was rehomogenized in solution A containing 80 mM-NaCl to give a volume equal to that of the original homogenate. All the above manipulations were performed at 4 °C. Both fractions were frozen in liquid N_2 and stored at −75 °C without loss of hexokinase activity.

For solubilization of particle-bound hexokinases, digitonin was added to the resuspended pellet fraction to a concentration of 2 mg/mg of protein. The sample was left in an ice bath for 15 min with constant stirring and further homogenized in a Dounce homogenizer with ten strokes of a tight-fitting pestle. The digitonin-treated suspension was centrifuged at 149000 $g_{av.}$ for 60 min at 4 °C. The supernatant containing solubilized protein was removed and the residual pellet was suspended in solution A containing 80 mM-NaCl as above.

Brain, epididymal adipose tissue and liver were taken from adult Wistar rats given a 20% (w/v) glucose solution for 20 h before the experiments in addition to regular food pellets. Tissues were homogenized and cytosols prepared as described above for cultured cells.

Ion-exchange chromatography

DEAE-cellulose was equilibrated with a solution of 10 mM-Tris/HCl, pH 7.0, 1 mM-EDTA, 1 mM-$MgCl_2$, 2 mM-dithiothreitol, 10% (w/v) glycerol (solution B) also containing 20 mM-NaCl. Columns with 2 ml of packed resin were used for chromatography of cytosol samples containing 20–40 mg of protein. Before loading, cytosols were diluted with solution B to lower the NaCl concentration to 20 mM. Elution of the adsorbed protein was accomplished by a linear gradient of NaCl concentration from 20 to 320 mM, made up in 60 ml of solution B; 1 ml fractions were collected and assayed for protein content and hexokinase activity. Chromatography was done at 4 °C.

DEAE-Sephadex was equilibrated with solution B containing 120 mM-NaCl. Fractions containing glucokinase after the DEAE-cellulose step were pooled, mixed with solution B to give a NaCl concentration of 120 mM and loaded on a column of 3 ml of packed DEAE-Sephadex. Retained protein was eluted by a gradient of NaCl from 120 to 400 mM in 60 ml of solution B; 1 ml fractions were collected.

Samples of partially purified isoenzymes destined for kinetics and immunotitration experiments were supplemented with bovine serum albumin to a final concentration of 0.2% (w/v), frozen in liquid N_2 and stored at −75 °C until analysis. Samples for electrophoresis and immunoblotting were concentrated by ultrafiltration and stored at −75 °C without added albumin. Protein assays were done by the Bradford [19] procedure.

Hexokinase assay

Hexokinase activity was assayed in a system using glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* as coupling enzyme. The reduced coenzyme NADH was measured fluorimetrically. The assay system was designed as described by Trus *et al.* [13], except that the volume of the reaction mixture was increased 2-fold. Samples were routinely assayed at 0.5 mM- and 100 mM-glucose, for determination of 'low-K_m' and total hexokinase activity respectively. One unit of activity was defined as the amount of enzyme that transforms 1 μmol of substrate/min at 30 °C. Detailed saturation kinetics for the various isoenzymes were established on the basis of rate measurements performed with at least seven distinct glucose concentrations. Values for K_m and Hill coefficient (h) were derived by standard computation [20]. Immunotitration with antiserum to glucokinase was performed as previously described [12].

Gel electrophoresis and immunoblotting

SDS/polyacrylamide-gel electrophoresis was performed as described by Laemmli [21]. High-resolution two-dimensional gel electrophoresis was done essentially by the method of O'Farrell [22]. However, the zwitterionic detergent CHAPS was used in place of Nonidet P-40 for the isoelectric-focusing dimension, as recommended by Perdew *et al.* [23]. Protein samples were prepared for electrophoresis by freeze-drying or trichloroacetic acid precipitation.

Electrophoretic transfer of proteins from gels to nitrocellulose membranes and immunoblotting with antibodies to rat liver glucokinase were performed exactly as described previously [12].

Peptide mapping

Limited proteolysis in the presence of SDS to generate peptide maps was performed as described by Cleveland *et al.* [24]. The proteinases used were chymotrypsin and papain. Digestion was conducted as specified in the legend to Fig. 5. The proteolytic products were resolved by gel electrophoresis and transferred to a nitrocellulose membrane. The peptides derived from glucokinase were revealed by immunoblotting with antibodies to glucokinase.

Materials

Culture medium was purchased from Amimed (Basel, Switzerland) and fetal-calf serum from Gibco (Grand Island, N.Y. U.S.A.). DEAE-cellulose and DEAE-Sephadex were products of Whatman (Maidstone, Kent, U.K.) and Pharmacia (Uppsala, Sweden) respectively. Biochemicals for enzyme assay were obtained from Boehringer (Mannheim, Germany). The detergent CHAPS, chymotrypsin (type VII) and papain (type IV) were bought from Sigma (St. Louis, MO, U.S.A.). Nitrocellulose membranes were from Amersham International (Amersham, Bucks., U.K.).

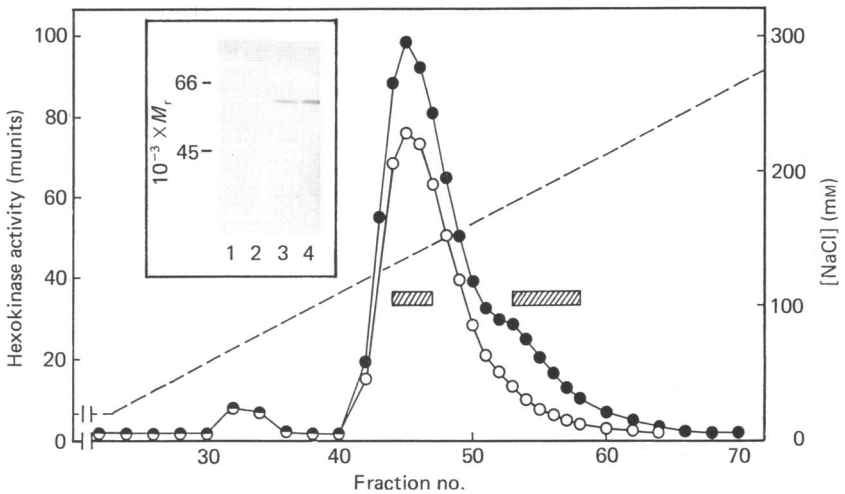

Fig. 1. DEAE-cellulose chromatography of cytosolic hexokinases from RIN-m5F cells

Hexokinase activity was measured at 0.5 mM-glucose (○) and 100 mM-glucose (●): ————, gradient of NaCl concentration in elution buffer. Methods are described in the Experimental section. The inset shows a glucokinase immunoblot of fractions shown by the horizontal hatched bars. Lanes 1 and 2, 10 and 20 μg of protein from pooled fractions 44–46; lanes 3 and 4, 10 and 20 μg of protein from pooled fractions 53–58. M_r markers are bovine serum albumin and ovalbumin. The gel was 11% polyacrylamide.

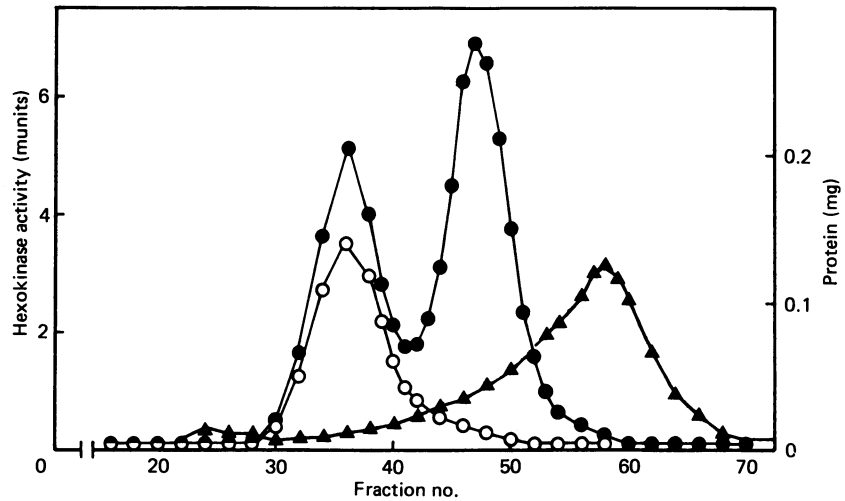

Fig. 2. Purification of glucokinase from RIN-m5F cells by DEAE-Sephadex chromatography

Fractions shown by the right-hand hatched bar in Fig. 1 were pooled and chromatographed on DEAE-Sephadex as described in the Experimental section. The linear NaCl concentration gradient from 120 to 400 mM was started at fraction 15 and terminated at fraction 76. Hexokinase activity was measured at (○) 0.5 mM- and (●) 100 mM-glucose; ▲, protein content.

RESULTS

Hexokinases of RIN-m5F cell cytosol

The pattern of hexokinases in the cytosol of RIN-m5F cells was analysed by ion-exchange chromatography on DEAE-cellulose. As shown in Fig. 1, this procedure resolved two peaks of enzyme activity and a third component displayed as a shoulder on the trailing edge of the second peak. To identify these isoenzyme forms, parallel experiments were done with rat brain and adipose-tissue cytosols. The major hexokinase of brain cytosol, i.e. hexokinase type I, was eluted at the same position as the first peak from RIN-m5F cells. Similarly, the major hexokinase of epididymal fat-pads, i.e. hexokinase type II, co-eluted with the second peak of the RIN-m5F cell chromatogram (results not shown). On this basis, the first and second peaks in the chromatogram of Fig. 1 were identified as hexokinases type I and II respectively.

The abundant RIN-m5F isoenzyme identified as hexokinase type II (left-hand horizontal bar in Fig. 1) was further characterized by kinetic experiments. We measured a K_m for glucose of 0.15 mM for the insulinoma-cell enzyme, as well as for hexokinase type II from adipose tissue. This value is in agreement with previous data for the adipose-tissue enzyme [2,5]. It is noteworthy that the glucose concentration of 0.5 mM used in the standard assay to measure 'low-K_m'

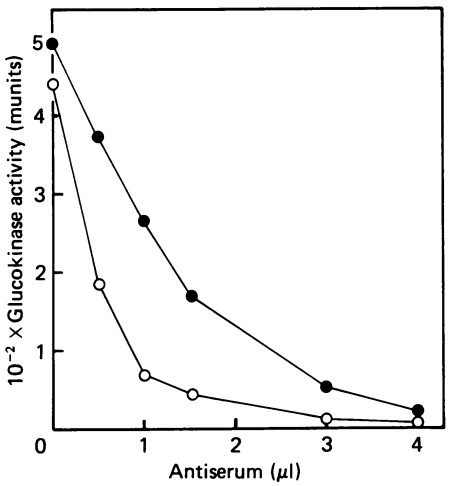

Fig. 3. Immunotitration of glucokinase

Fixed amounts of glucokinase purified by DEAE-cellulose and DEAE-Sephadex chromatography were made to react with increasing volumes of antiserum to rat liver glucokinase at 37 °C for 30 min and 4 °C for 1 h. Glucokinase activity remaining after the antigen-antibody reaction is shown for the enzyme from RIN-m5F cells (●) and from rat liver (○). Incubation with pre-immune serum resulted in no loss of enzyme activity (results not shown).

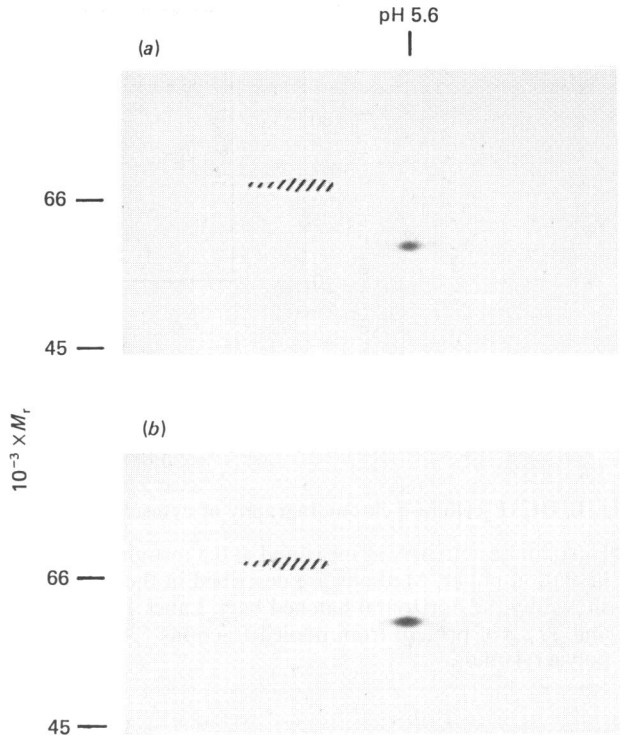

Fig. 4. Two-dimensional gel electrophoresis and immunoblotting of glucokinase

Enzyme from rat liver (*a*) and RIN-m5F cells (*b*) was partially purified by chromatography on DEAE-cellulose and DEAE-Sephadex. In each case protein samples containing 0.3 munits of glucokinase activity were precipitated with trichloroacetic acid and loaded on the first-dimension gel. Total protein load on the two gels was made equal by adding bulk protein from rat brain cytosol to the liver sample. [^{14}C]Methylated bovine serum albumin was added as marker to the two samples and detected by autoradiography of the blots (hatched areas in *a* and *b*). Photographs show only the relevant area of the blots. Second-dimension gels were 11% polyacrylamide. M_r markers are bovine serum albumin and ovalbumin.

hexokinase activity is insufficient to saturate hexokinase type II (see the difference in rates noted at 0.5 mM- and 100 mM-glucose across the second peak in Fig. 1). Hexokinase type II will therefore contribute to 'high-K_m' glucose-6-phosphotransferase activity in unfractionated cell extracts.

The third component in the chromatogram of Fig. 1 (right-hand horizontal bar) exhibited a high ratio of enzyme activity at 100 mM-glucose to that at 0.5 mM, suggesting that it might represent glucokinase. To verify this point, the fractions of interest were pooled and tested for the presence of glucokinase by immunoblotting with antibodies to rat liver glucokinase. As shown in the inset of Fig. 1, these fractions displayed a single immunoreactive polypeptide with apparent M_r 56500, which was absent from earlier fractions. This band co-migrated with authentic rat liver glucokinase (not shown here, but see below and [12]), providing strong evidence for the presence of this isoenzyme in RIN-m5F cells.

The putative glucokinase protein was further purified by chromatography on DEAE-Sephadex. As shown in Fig. 2, this step allowed us to obtain glucokinase essentially free of hexokinase type II. The enzyme at this stage of purification was characterized for its catalytic and structural properties in the following experiments.

Kinetics and immunotitration

The glucose concentration for half-maximal velocity of the RIN-m5F cell glucokinase was 5.6 mM, cf. 5.3 mM for hepatic glucokinase under the same experimental conditions. The enzyme from the cultured cells and from liver showed sigmoid saturation kinetics with glucose, with Hill coefficients at 5 mM-MgATP concentration of 1.54 and 1.48 respectively. These data are in full agreement with the values published by Storer & Cornish-Bowden [25] for hepatic glucokinase.

Antibodies to rat liver glucokinase abolished the activity of RIN-m5F cell glucokinase, as shown in Fig. 3. However, the potency of the antibodies toward the enzyme from the insulinoma cells appeared to be consistently decreased as compared with their activity against liver glucokinase. This minor difference in slope might reflect a slight decrease in catalytic activity per enzyme molecule in the sample from the RIN-m5F cells, possibly occurring during isolation or storage.

Molecular size and net charge

High-resolution two-dimensional gel electrophoresis followed by immunoblotting were used to compare glucokinase in RIN-m5F cells and liver with respect to pI and M_r. The relevant area of the immunoblots is shown in Fig. 4. The blots were aligned accurately by using the marker provided by [^{14}C]methylated bovine serum albumin that was added to the samples before electrophoresis and detected by autoradiography (hatched spots in Fig. 4). Both immunoblots revealed a well-focused spot corresponding to a protein with apparent pI 5.6 and M_r 56500. In previous work from this laboratory, liver glucokinase was shown to migrate as

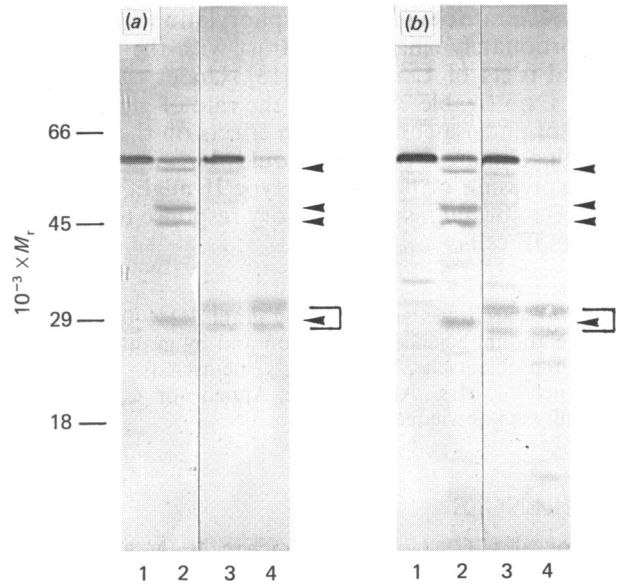

Fig. 5. Peptide mapping of glucokinase

Enzyme from rat liver (*a*) and RIN-m5F cells (*b*) was partially purified by chromatography on DEAE-cellulose and DEAE-Sephadex. Samples containing 1.5 munits of glucokinase were used for each digestion condition. Bulk protein from rat brain cytosol was added to the liver samples such that total protein content was the same as for RIN-m5F samples (15 μg of protein). Proteolysis was performed in solution before loading of samples on the gel. Gel electrophoresis was in a 12.5% polyacrylamide gel. Transfer of the proteolytic products to nitrocellulose and immunochemical detection of glucokinase-derived peptides were performed by the immunoblotting procedure described in the Experimental section. Lanes: 1, control sample without proteinase; 2, digestion with 0.4 μg of chymotrypsin for 60 min at 37 °C; 3, digestion with 0.01 μg of papain for 15 min; 4, digestion with 0.01 μg of papain for 60 min. M_r markers were bovine serum albumin, ovalbumin, carbonic anhydrase and β-lactoglobulin.

two closely spaced isoforms, with pI 5.64 and 5.54 [12]. We ascribe this difference to the use of Nonidet P-40 as detergent in the previous experiments and of CHAPS in the present ones. The latter detergent was shown to lessen streaking of proteins during isoelectric focusing, especially in the acid part of the pH gradient [23].

Peptide mapping

Peptide maps of glucokinase without prior purification of the protein to homogeneity were obtained by combining the techniques of limited proteolysis in presence of SDS, Western transfer and immunochemical detection of relevant peptides with the anti-glucokinase antibodies (Fig. 5). This approach resulted in the identification of four typical peptides after chymotrypsin digestion (arrowheads in Fig. 5) and two typical peptides after papain digestion (bracket in Fig. 5). A third immunoreactive peptide, with M_r 23000 after digestion with papain, was visible on direct examination of the blots. With both proteolytic enzymes, the peptides bearing glucokinase epitopes were identical in samples from RIN-m5F cells and liver, suggesting the presence of one and the same gene product in the two cell types.

Subcellular distribution of hexokinase isoenzymes

An impressive finding in the course of this work was the large amount of hexokinase associated with the pellet after high-speed centrifugation of RIN-m5F cell homogenates. As shown in Table 1, only one-fifth of total cellular hexokinase was cytosolic, the remainder being found in the high-speed pellet. Approximately two-thirds of particulate hexokinase activity were recovered in soluble form after digitonin treatment of the pellet. Hexokinase type II accounted for 90% and hexokinase type I for 10% of the enzyme activity in the digitonin extract, as determined by DEAE-cellulose chromatography (results not shown). Moreover, the hexokinase remaining insoluble after detergent treatment had a measured K_m for glucose of 0.15 mM, consistent with the prevalence of hexokinase type II in this fraction also. Therefore we concluded that hexokinase type II represented the vast majority of insoluble hexokinase activity in RIN-m5F cells. Although the precise subcellular localization of this particle-bound hexokinase was beyond the scope of this work, preliminary results indicated that it sedimented with a crude mitochondrial fraction during differential ultracentrifugation.

DISCUSSION

The data presented here show that insulinoma cells contain an enzyme with the same kinetic, chromatographic and structural properties as rat liver glucokinase. Two-dimensional gel electrophoresis demonstrated that the enzymes from both sources had identical net charge and molecular size. More definitively, peptide mapping revealed a common primary structure, at least within the

Table 1. Subcellular distribution of hexokinases in RIN-m5F cells

Homogenization of cells, high-speed centrifugation and hexokinase assay were performed as described in the Experimental section. Data are normalized to 1 mg of total cellular protein, and are given as means ± S.E.M. for nine experiments.

Cell fraction	Protein content (mg)	Hexokinase activity (munits)	
		At 0.5 mM-glucose	At 100 mM-glucose
Homogenate	1.00	39.3 ± 1.7	49.7 ± 2.6
Cytosol	0.29 ± 0.01	5.9 ± 0.5	8.8 ± 0.7
Particulate fraction	0.70 ± 0.03	31.1 ± 2.1	38.6 ± 2.7

limits of the present methodology. Therefore we conclude that the gene coding for liver glucokinase is expressed in the RIN-m5F cell line. On the basis of immunoblotting data as well as chromatography and enzyme assay, the amount of glucokinase relative to total cytosol protein in these cells was comparable with that in islets of Langerhans, i.e. about one-twentieth of that in the liver of glucose-fed rats [12].

The RIN-m5F cells are the first established cell-culture line known to express the glucokinase gene, albeit at a low level. Weinhouse and collaborators showed previously [8] that tumoral transformation of the liver cell could result in the loss of glucokinase; thus, this enzyme was absent from poorly differentiated rat hepatomas and Novikoff hepatoma ascites cells. Whether glucokinase could be detected in some of the well-differentiated liver cell lines currently available, by using the highly sensitive and specific procedures employed here, is an open question.

The first evidence for the presence of glucokinase in islets of Langerhans and rat insulinomas was provided by Matschinsky and co-workers [26,27], who also suggested that glucokinase was a rate-limiting enzyme of glycolysis in the β-cell of the islet [13]. Since the glycolytic flux is known to be a major determinant of the rate of insulin secretion, glucokinase has been viewed as a key regulatory element for the control of insulin secretion by glucose and termed the 'glucose sensor' of the β-cell [14]. Direct evidence at the molecular level for the expression of the glucokinase gene in insulin-producing cells is given in the present paper. Yet, it should be noted that RIN-m5F cells do not display an increase in insulin secretion when exposed to increasing glucose concentrations in the 2–30 mM range, in contrast with normal islets [18]. Therefore, expression of glucokinase may be a necessary condition for glucose-induced insulin release, but it is clearly not a sufficient one.

A striking feature of the RIN-m5F cells is their high content of hexokinase type II. This isoenzyme appears to be missing from the parent tissue, the pancreatic islet [28]. The occurrence in tumour cells of a hexokinase type not found in the tissue of origin is not without precedent. In Novikoff hepatoma cells, concomitantly with the loss of glucokinase mentioned above, hexokinases type II and III were found to become prevalent, whereas these two isoenzymes are barely detectable in normal liver [8]. More recently, Pedersen and associates reported that a fraction of the hexokinase activity in a number of heptomas was associated with mitochondria, apparently as the result of an interaction between the hexokinase molecule and a specific protein of the mitochondrial outer membrane termed 'porin' [9,29,30]. Both overexpression of atypical hexokinase forms and binding to mitochondria are considered as important mechanisms for sustaining the high glycolytic rates of malignant hepatomas [9,29]. The abundance of hexokinase type II and its association with a particulate subcellular fraction in RIN-m5F cells, together with the exaggerated rate of glycolysis noted previously by Halban *et al.* [16], are reminiscent of the earlier findings in hepatomas.

From the physiological standpoint, the vast excess of hexokinase type II over glucokinase activity makes it highly unlikely that glucokinase could act as a rate-limiting enzyme in RIN-m5F cells. Even assuming a profound inhibition of the hexokinase by intracellular glucose 6-phosphate, the contribution of glucokinase to the overall rate of glucose phosphorylation would remain proportionately small, in contrast with the situation in normal islets of Langerhans [13]. Under these circumstances, no sizable changes in the rate of glycolysis as a result of glucose-concentration effects on the velocity of the glucokinase reaction should be expected. Thus overexpression of hexokinase type II might explain the lack of a normal insulin-secretory response to glucose in RIN-m5F cells.

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