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High-resolution two-dimensional polyacrylamide gel electrophoresis reveals a glucose-response protein of 65 kDa in pancreatic islet cells

(protein biosynthesis/glucose homeostasis/pancreatic β cell/insulin secretion/glucose metabolism)

HEATHER WEIK COLLINS, CAROL BUETTGER, AND FRANZ MATSCHINSKY

Department of Biochemistry and Biophysics and the Diabetes Research Center of the University of Pennsylvania School of Medicine, Philadelphia, PA 19104

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ABSTRACT **High-resolution two-dimensional PAGE was used to search for glucose-response proteins in isolated pancreatic islets that were labeled with [^{35}S]methionine at ambient glucose concentrations of 0–18 mM. A 65-kDa protein, isoelectric focusing point of approximately 6.6–7.0, was discovered that showed at least a 20-fold stimulation of radiolabeling when glucose in the labeling medium was increased from 3 to 18 mM, in contrast to a 2.5-fold enhancement of label incorporation into total islet proteins. This 65-kDa protein is evident after 30 min of labeling with 18 mM glucose and is preferentially synthesized compared to its nearest neighbors after both 30 and 60 min of labeling. Glucose induction of the 65-kDa protein was virtually blocked by D-*manno*heptulose. Glucose induction of this 65-kDa protein is in practically all aspects comparable to glucose induction of insulin and glucokinase in pancreatic β cells. A working hypothesis is developed proposing that glucose-response proteins or "glucospondins" are pivotal constituents of pancreatic islet cells and that their discovery and exploration promise new insights into normal and pathological islet cell function.**

Physiologically elevated blood glucose influences pancreatic β cells thoroughly. Insulin release is stimulated (1) and insulin biosynthesis is augmented within minutes after glucose levels increase (2–4). If hyperglycemia persists for hours or even longer β cells become sensitized to glucose and ultimately they respond with hypertrophy and hyperplasia (5). Those multifarious β-cell responses promoted by high glucose can be expected to be associated with the activation of known and proposed glucose-response genes and the enhanced biosynthesis of corresponding glucose-response proteins. Insulin (6, 7) and glucokinase (8) are examples of established β-cell glucose-response proteins.

Considering current knowledge, it is reasonable to assume that enhanced β-cell glucose metabolism is required for glucose to evoke those effects, that the glucose concentration dependency curves of those diversified responses show much the same sigmoidal shape, with thresholds between 3 and 5 mM and maximal potential at 15–20 mM glucose (9), and that the responses are inhibitable by *manno*heptulose, a specific blocker of glucose phosphorylation and metabolism (10, 11). It can also be expected that many of the glucose effects can be elicited by the metabolic fuel analogue α-ketoisocaproate (KIC). This has been documented for insulin release and biosynthesis (12) but has not been explored for other adaptive responses of the β cells, for example, glucose-induced hypertrophy and hyperplasia with the associated altered gene expression and protein biosynthesis.

High-resolution two-dimensional (2-D) polyacrylamide gel electrophoresis (PAGE) of radioactively labeled tissue affords a possible approach to explore the biosynthesis of 1000–2000 proteins per sample (13). When combined with accurate calibration and computerized quantitative analysis, this technique can reliably assess radioactive label incorporation over two to three orders of magnitude. We have begun to apply this technique to study the role of protein biosynthesis in the adaptive responses of β cells to glucose stimulation, and we have discovered a 65-kDa protein that is synthesized in a manner closely resembling glucose-induced insulin biosynthesis and release. The 65-kDa protein, which we refer to as "glucospondin" (GSP) 65 (see *Discussion*), is described here as a prototypical glucose-response protein and is used to establish criteria that could define glucose-response proteins in pancreatic β cells and possibly in other tissues.

METHODS

Rat islets were prepared by collagenase (Serva) digestion as previously described (14). For the purpose of labeling proteins with [^{35}S]methionine, 100 isolated islets were placed into wells of 24-well plates and suspended in 1–2 ml of Hepes-buffered Hanks' solution. Two samples were concurrently processed per plate. The Hanks' Hepes buffer was removed and each sample was washed twice with 250 μl of RPMI 1640 medium (Flow Laboratories) containing 10% fetal calf serum (Flow Laboratories) plus secretagogue but without methionine. After the second wash, 200 μl of the above medium was added to each well followed by 50 μl of the same medium containing 0.5 mCi (1 Ci = 37 GBq) of [^{35}S]-methionine. The plate was placed in a modular incubator chamber, gassed with a mixture of 95% air and 5% CO_2 for 45 sec, sealed, and placed in a 37°C incubator, usually for 2 hr. After the incubation, the plate was removed from the chamber, the incubation medium was removed, and the islets were then washed twice with 250 μl of ice-cold phosphate-buffered saline. After removal of the second wash fluid, 200 μl of boiling 0.3% sodium dodecyl sulfate containing 5% (vol/vol) 2-mercaptoethanol was added to the islets. The plate was placed in a boiling water bath for 1 min and the islets were mechanically disrupted with a 100-μl Hamilton syringe with a fine barrel by repeated aspiration and ejection. When all islets had been completely homogenized, 20 μl of a solution containing DNase at 1 mg/ml and RNase at 500 μg/ml was added, and the homogenate was mixed and allowed to digest for 1 min at room temperature. The sample was transferred into a 1.5-ml Microfuge tube and frozen in liquid nitrogen.

In preparation for 2-D gel analysis, samples were lyophilized and reconstituted in 200 μl of isoelectric focusing buffer, which contains 9.5 M urea (Schwarz/Mann), 2%

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Abbreviations: KIC, α-ketoisocaproate; 2-D, two-dimensional; GSP, glucospondin.

Nonidet P-40 (Accurate Chemicals, Westbury, CT), 100 mM dithiothreitol (Calbiochem), and 2% carrier Ampholines (BDH, Resolyte 4–8). If needed, samples were diluted so that a 10-μl aliquot contained approximately 400,000 cpm.

The 2-D gel electrophoresis was performed after the method of Garrels (15). Ten microliters of each sample was loaded into a capillary tube (0.8 mm diameter, 20 cm long) containing 2.9% acrylamide (all other electrophoresis reagents were from Bio-Rad), 2% Nonidet P-40, 9.5 M urea, and 2% Ampholines, pH 4–8. The proteins were focused for 15,000 V·hr. After extrusion, most gels were cut approximately 9 cm from the basic end and the basic portions of eight samples were loaded onto the 10% acrylamide slab and electrophoresed at 110 mA. Processing only half the isoelectric focusing gels greatly increased the efficiency of the procedure.

Gels were processed for fluorography, rinsed, and dried onto filter paper with a calibration strip of proteins labeled with [^{35}S]methionine (13). Four exposures at −70°C were made for each gel.

X-ray films were scanned with an Optronix P-1000 scanner at 200-μm resolution in the x and y directions. The images were then calibrated and merged by using the PDQUEST spot detection algorithms (13, 15). After this, the gel images for each set of experiments were gathered into a matchset, which contains a reference gel and the sample gel images. Images were then compared to the fluorograms as a check. For purposes of the present study, only the six spots nearest to the glucose-response protein described in this report were matched to the standard image. Following this, all quantitation of dpm per protein was corrected to dpm incorporated per protein per islet.

RESULTS

Total protein synthesis of isolated pancreatic islets as measured by radiolabeling of trichloroacetic acid-precipitable material with [^{35}S]methionine was increased approximately 150% as the glucose concentration of the labeling medium was increased from 3 to 18 mM (Table 1 and Fig. 1). Comparable stimulations of total protein synthesis by high glucose were observed in a time dependency experiment of [^{35}S]methionine incorporation (Fig. 3*A*) and in an experiment designed to explore whether increased metabolism of glucose might be the basis of augmented protein synthesis (Table 3). Thus, general islet protein metabolism operated in a predicted manner as a result of the experimental manipulation in this study.

The long-term objective of this investigation is to search for protein synthesis changes in response to metabolic fuel secretagogues by using the powerful technique of high-resolution 2-D PAGE of proteins coupled with computerized image analysis and quantitation of data. A typical fluorogram of a 2-D gel prepared from isolated islets labeled for 2 hr with [^{35}S]methionine in the presence of 18 mM glucose is shown to illustrate the approach (Fig. 2). On visual inspection (without the aid of the comprehensive computerized image analysis) and comparing fluorograms of 2-D PAGE gels from islets labeled in low (3 mM) and high (18 mM) glucose, the enhanced labeling of a slightly acidic protein, approximately 65 kDa, was readily apparent. The 65-kDa protein was not easily discerned in gels from islets incubated in 3 mM glucose (not shown). Attention therefore focused on this striking observation and experiments were designed to better characterize the phenomenon.

The results in Table 1 and Fig. 1 show that the threshold for enhanced labeling of the 65-kDa protein with [^{35}S]methionine lies between 3 and 6 mM glucose and that a 16- to 20-fold stimulation was achieved at 18 mM glucose, in comparison to the baseline of 3 mM glucose. Differential stimulation of the

Table 1. Concentration dependency of glucose stimulation of total protein and GSP 65 biosynthesis and differential glucose stimulation of GSP 65 as compared to total protein biosynthesis

Glucose, mM	GSP 65 labeling, dpm/islet	Labeling of total protein, dpm/islet	Differential glucose effect on GSP 65 (relative to 3 mM glucose)
		Experiment 1	
0	5.12	71,708	0.81
3	10.31	116,127	1.00
6	31.68	125,511	2.84
9	98.16	227,947	4.86
12	140.64	309,119	5.13
18	158.39	290,496	6.15
		Experiment 2	
0	8.97	86,954	1.59
3	6.32	97,407	1.00
6	24.19	138,442	2.70
9	67.05	196,685	5.25
		Experiment 3	
0	12.43	113,250	1.73
3	11.43	179,172	1.00
6	84.68	265,195	5.01
9	287.37	481,218	9.35
12	209.93	341,356	9.62
18	203.37	335,944	9.51

In each experiment the results of groups of islets separately labeled are presented. A graphic interpretation of the results is shown in Fig. 1.

65-kDa protein labeling as compared with the stimulation of overall protein labeling was 6.2- to 9.5-fold under standard conditions for labeling. Half-maximal stimulation was achieved at 9 mM glucose. The outcome of the time dependency studies (Fig. 3) offers a second argument for designating the 65-kDa protein a glucose-response protein. The basal rate of labeling total protein in the presence of 3 mM glucose was substantial and reached an average of 275,000 dpm per islet in 3 hr, and increasing the glucose in the labeling medium

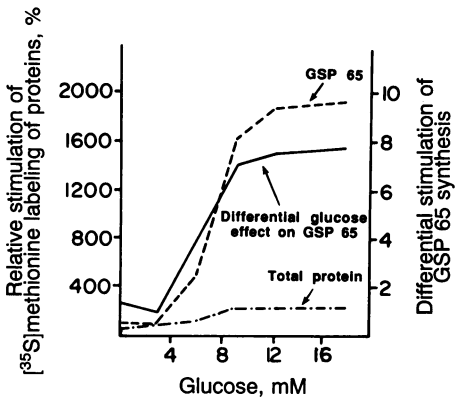

FIG. 1. Concentration dependency of glucose stimulation of total protein synthesis, GSP 65 synthesis, and the specific stimulation of GSP 65. Islets were labeled under standard conditions and the basic segments of the isoelectric focusing gels were further processed to separate proteins in the second dimension. Results are expressed as the mean of three (0–9 mM) or two (12–18 mM) experiments. The baseline of 100% for both GSP 65 synthesis and total protein synthesis was obtained at 3 mM glucose. Baseline labeling was 9.35 ± 1.90 (±SEM, n = 3) and 130,902 ± 24,733 (n = 3) dpm per islet for GSP 65 and total acid-precipitable material, respectively. The differential stimulation of GSP 65 synthesis was obtained from the ratio of percent stimulation for GSP 65 divided by percent stimulation for total acid-precipitable material. To enhance the clarity of the figure, standard errors were not included. The graph is based upon the data presented in Table 1.

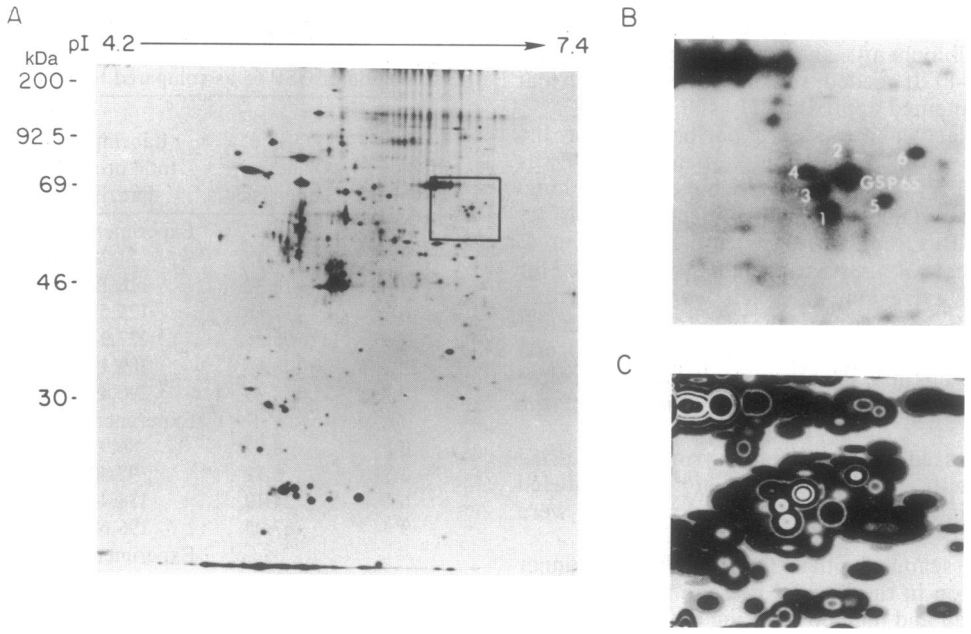

FIG. 2. Fluorogram of 2-D gel from normal rat islet lysate. Freshly isolated islets were labeled for 2 hr in the presence of 18 mM glucose. The entire isoelectric focusing gel was loaded onto the second-dimension slab. The slab was run under standard conditions and the dried gel was exposed to the film at −70°C for approximately 12 days. (*A*) Complete gel. (*B*) Magnification of spot constellation including GSP 65. (*C*) Computer image of *B*.

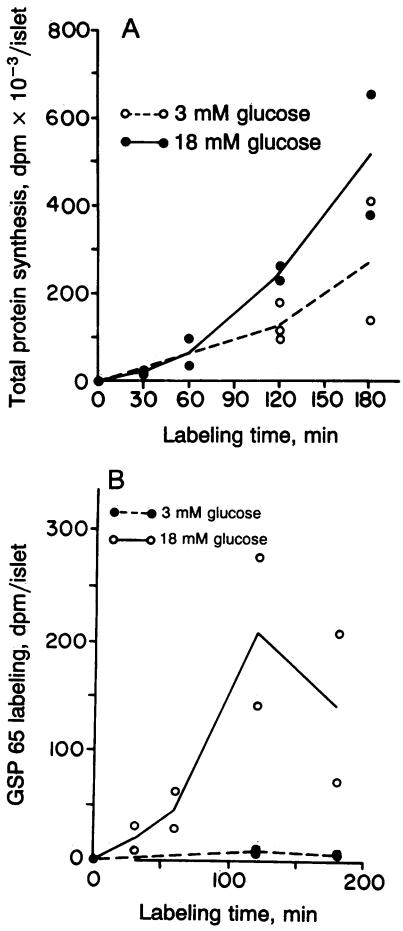

FIG. 3. Effect of time of labeling on total protein synthesis (*A*) and GSP 65 synthesis (*B*). Individual points and lines through the means of two experiments are presented. Labeling at 30 and 60 min was not measured for 3 mM glucose.

to 18 mM doubled the rate of [^{35}S]methionine to an average of 525,000 dpm per islet in 3 hr, on average a less than 2-fold stimulation. In contrast, the stimulation of labeling the 65-kDa protein by high glucose was 13- to 28-fold at its maximal rate at the 120-min time point, while labeling at 3 mM glucose was barely detectable. The time course study also revealed that the stimulation of labeling of the 65-kDa protein was clear at 30 min, a time when six neighboring proteins showed practically no incorporation of [^{35}S]methionine (Table 2). Thus the 65-kDa protein stands out by effective initiation of its biosynthesis within minutes after the switch to 18 mM glucose.

Insulin release and synthesis are clearly tied to glucose metabolism and both can be blocked by the presence of D-*manno*heptulose, a glucose phosphorylation inhibitor. An experiment was therefore designed to test if the synthesis of the 65-kDa protein was affected by *manno*heptulose. This was indeed the case, as clearly shown in Table 3 and Fig. 4. *Manno*heptulose virtually blocked the glucose-induced labeling of the 65-kDa protein. It is remarkable that *manno*heptulose had little influence on the glucose stimulation of total protein synthesis. It was observed that KIC at 15 mM also enhanced the labeling of the 65-kDa protein by about 6-fold and that *manno*heptulose inhibited this effect by about 30%. It should be realized that the incubation medium contained about 0.5 mM glucose in addition to the ketoacid. *Manno*heptulose can be expected to interfere with synergistic actions of the two fuels, thus explaining the 30% reduction in the effect of the ketoacid. The outcome of this experiment is strikingly illustrated by Fig. 4.

DISCUSSION

We have adopted the working hypothesis that pancreatic islet cell glucose-response proteins or "glucospondins" (GSPs) are characteristic and functionally essential molecular constituents of these endocrine cells. GSPs are defined as glucose-inducible or -repressable proteins that are crucial for the vital glucostat role of pancreatic endocrine cells. The 65-kDa protein is described here as the prototypical glucose-response protein and is used to establish criteria that define

Table 2. [^{35}S]Methionine incorporation into GSP 65 and neighboring proteins during short-term incubations in 18 mM glucose

	[^{35}S]Methionine incorporation, dpm per islet							
	30 min		60 min		120 min		180 min	
Protein	a	b	a	b	a	b	a	b
1	6.78	1.62	9.80	34.88	105.16	145.94	140.71	306.36
2	4.15	0.32	1.49	12.20	35.89	43.56	38.60	19.89
3	0.22	0.81	14.27	33.21	9.45	215.21	170.60	229.44
4	3.50	1.46	3.83	15.46	64.86	64.47	68.49	185.67
5	2.40	0.81	5.33	19.01	39.67	76.24	2.49	15.91
6	0.66	1.62	1.28	4.60	65.46	24.83	43.58	311.66
GSP 65	31.05	8.43	29.18	62.67	278.33	142.45	73.47	210.87

Results of two separate experiments (a and b) are shown. Total protein synthesis is shown in Fig. 3*A*.

glucose response proteins in pancreatic β cells. Four criteria need to be met for a protein to qualify as a pancreatic islet cell glucose-response protein:

(*i*) The rate of biosynthesis and/or degradation must be positively or negatively regulated by glucose in a physiological concentration-dependent manner with a threshold of 3–5 mM, half-maximal stimulation at 8–10 mM, and maximal potency of glucose at 15–20 mM.*

(*ii*) To qualify as a specific glucose-response protein a protein requires significant differential stimulation or inhibition compared to general protein biosynthesis. In view of the established approximately 2-fold stimulation of general protein biosynthesis in islet tissue by high glucose, a maximal differential effect of glucose equal to or larger than 3-fold is presently chosen (somewhat arbitrarily) as a practical guide.

(*iii*) The effect of glucose on protein synthesis and/or degradation must be blocked by *manno*heptulose.

(*iv*) The effect of glucose on protein synthesis should be mimicked by KIC but the action of this fuel should be largely resistant to *manno*heptulose.

The 65-kDa protein described in this report is an islet GSP according to the criteria defined above and is therefore called GSP 65. The glucose dependency curve of its stimulated biosynthesis is impressively sigmoidal, with a threshold between 3 and 6 mM and a maximum at 18 mM glucose. Maximal differential glucose stimulation of its biosynthesis is approximately 8-fold. The effect of glucose is virtually blocked by *manno*heptulose, and KIC mimics the action of glucose but is much less susceptible to *manno*heptulose inhibition. It is likely that the protein is expressed in the β cells and/or δ cells because of its glucose dependency curve, which is characteristic for those cells (16, 17). Presumed α-cell glucose-response proteins can be expected to show a glucose-response curve with a threshold of 1 mM glucose or less and a saturation level of 5–6 mM glucose (9). The biochemical basis of glucose induction and the physiological role of GSP 65 are not known. GSP 65 appears to be a low-abundance protein because it is not demonstrable on silver-stained high-resolution gels loaded with about 50 μg of protein (data not shown), and its isolation and chemical characterization might therefore be a difficult task. It is not yet known whether GSP 65 might be related to or identical to the 64-kDa protein that has a similar pI and appears to play a role in the pathogenesis of type I diabetes (18, 19).

Two other β-cell glucose-response proteins as here defined are already known: insulin and glucokinase. In the case of insulin, all criteria above are fulfilled (2–4), and detailed molecular genetic research has revealed the biochemical basis of glucose regulation of insulin biosynthesis. The early increase of insulin biosynthesis caused by glucose is due to enhanced translation of existing proinsulin mRNA, and this effect is blocked by translation inhibitors (6). The increase seen during longer response to high glucose results from a stepped up proinsulin mRNA synthesis and is blocked by transcription inhibitors (4). The efficiency of binding of proinsulin mRNA to the rough endoplasmic reticulum is also improved by high glucose (7). The biochemical basis of glucose induction or activation of islet cell glucokinase is less well understood. What we know about this glucose-response protein arose primarily from enzyme activity measurements in islets isolated from hypo- and hyperglycemic animals (20) or from rat islets cultured at low or higher glucose (8). A 5-fold range of glucokinase specific activity was obtained in islets cultured in 30 as compared to 3 mM glucose (8). It appears from preliminary reports that regulation of glucokinase occurs primarily at the translational or post-translational

*Note that the glucose-response curve for α-cell glucose-response proteins can be expected to be shifted to the left, corresponding to the relatively high glucose sensitivity of these cells.

Table 3. Effect of *manno*heptulose on [^{35}S]methionine labeling of total protein and selected individual proteins stimulated by glucose or KIC

	[^{35}S]Methionine incorporation, dpm per islet									
			9.5 mM glucose				15 mM KIC and 0.5 mM glucose			
			Without *manno*heptulose		With 30 mM *manno*heptulose		Without *manno*heptulose		With 30 mM *manno*heptulose	
Protein	0.5 mM glucose									
1	23.3	28.4	47.8	59.2	43.5	46.6	54.5	65.2	45.4	72.9
2	11.3	11.5	14.7	29.7	17.2	22.5	22.1	10.8	16.8	6.3
3	29.2	30.5	42.8	70.9	65.6	89.4	51.9	60.1	46.9	63.6
4	13.6	10.1	22.2	27.5	17.9	23.7	32.0	20.6	17.9	23.4
5	18.6	18.1	31.9	39.3	33.5	46.6	37.2	38.9	32.6	34.1
6	10.7	10.4	44.9	40.6	22.1	28.9	39.4	22.7	15.4	13.9
GSP 65	7.3	14.1	109.0	88.6	28.3	12.8	67.1	67.2	46.1	45.0
Total protein	88,369	114,418	156,494	164,027	140,759	209,687	157,803	175,074	119,268	144,437

Results of individual experiments are shown.

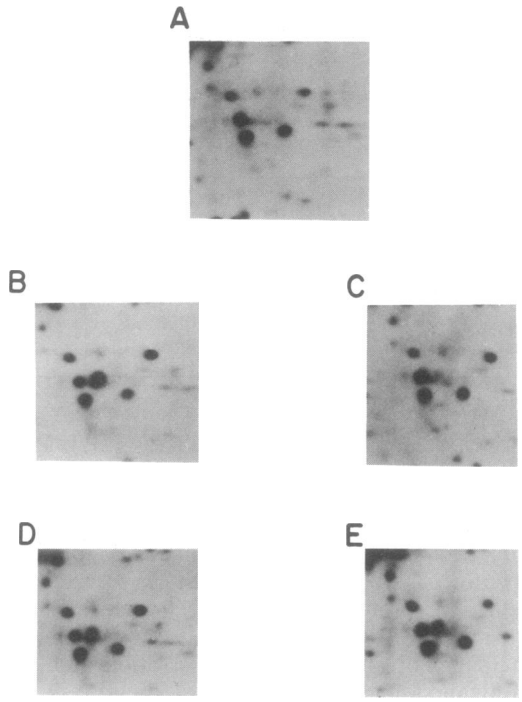

FIG. 4. Portions of fluorograms from [^{35}S]methionine-labeled islets focusing on GSP 65 and its close neighbor proteins labeled in the presence of 0.5 mM glucose (*A*), 9.5 mM glucose (*B*), 9.5 mM glucose plus 30 mM *manno*heptulose (*C*), 15 mM KIC plus 0.5 mM glucose (*D*), and 15 mM KIC plus 0.5 mM glucose plus 30 mM *manno*heptulose (*E*).

level, since pancreatic islet tissue glucokinase mRNA was found to be unaffected by a starvation and refeeding regimen (21). However, this experimental paradigm results in only small changes in ambient glucose (i.e., about 4 vs. 6 mM) and minor changes of pancreatic enzyme activity (i.e., a 30% decrease in starvation). Transcriptional influence of altered glucose in regulating β-cell glucokinase may thus have been overlooked. The physiological roles of insulin and glucokinase as the body's exclusive hypoglycemic hormone and as the pivotal pancreatic β-cell glucose sensor, respectively, suggest that those proteins are positively regulated by the ambient glucose level—i.e., that they possess the characteristics of glucose-response proteins or GSPs.

The scope of this collection of data is limited in three ways. First, the 2-D gel technique is limited in the actual number of proteins resolved per sample based upon the limited pH ranges for ampholytes and gel matrix composition (13). The second limitation involves the screening parameters for each sample. To facilitate sample processing, the pH range loaded onto the second-dimension gel was reduced and the computer analysis focused solely on GSP 65 and its nearest neighbors. To detect GSPs of other molecular weights and pI values, the islet glucose concentration dependency curve study needs to be repeated using broader pI ranges in the first dimension combined with a different matrix composition in the second dimension. Finally, the focus of this study was upon proteins that respond to glucose with a large increase in synthesis over a short period of time. Proteins with a long lag time until initiation of synthesis will not be detected in these experiments. Studies with longer labeling times would be needed to observe these changes.

It is, however, theoretically highly plausible and factually supported by the findings of this study and previous reports that the presence of glucose-response genes or proteins might be a biochemical design feature that enables pancreatic β cells (and the other endocrine cells of the pancreatic islet organ) to play their immensely important and complex role as glucostats maintaining the body's fuel homeostasis. Islet cells appear in this respect characteristically different from the cells of the liver (22), in which regulation of fuel metabolism is largely determined by hormonal effects, including the induction of hormone-response proteins—e.g., the enzymes glucokinase and phospho*enol*pyruvate carboxykinase induced by insulin and glucagon, respectively.

The identification and characterization of GSPs in addition to those that are already known (i.e., insulin, glucokinase, and GSP 65) seems to be a promising avenue for future islet cell research. The concept of GSPs appears teleologically attractive, prototypes are known, the technique of high-resolution 2-D PAGE is highly suitable to carry out a thorough search for them in scarce islet cell material, and the potential for developing in the course of this research new insights into islet cell function and growth appears substantial.

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