Glucose-stimulated protein phosphorylation in the pancreatic islet

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A glucose-dependent phosphorylation of a 68 kDa islet-cell protein was observed in islet-cell homogenates. In the presence of [γ - ^{32}P]ATP the protein was phosphorylated only in the presence of α -D-glucose; other sugars were ineffective. Activation of the phosphorylation was half-maximal at 0.34 mM-glucose, 7 μM -ATP and 0.3 mM-Mg $^{2+}$. Although the addition of glucose 6-phosphate in this design did not stimulate phosphorylation of the islet-cell protein, addition of glucose 6-phosphate to the radioactively labelled 68 kDa protein rapidly removed (chased) the ^{32}P label. The addition of presynthesized glucose 6-[^{32}P]phosphate phosphorylated the 68 kDa band in the islet-cell homogenate and also phosphorylated purified skeletal-muscle phosphoglucumutase. Phosphoglucumutase labelled thus by ^{32}P was indistinguishable from the islet-cell phosphoprotein on electrophoretic gels. The ^{32}P incorporated into both the islet-cell protein and the purified skeletal-muscle phosphoglucumutase was chased similarly by hexose phosphates. The purified phosphoglucumutase could also be phosphorylated by cyclic AMP-dependent protein kinase or by a mannoheptulose-insensitive process by the islet-cell cytosol. The phosphoenzyme formed thus was also dephosphorylated by D-glucose 6-phosphate and α -D-glucose 1-phosphate, suggesting that this may be a mechanism for generation of glucose 1,6-bisphosphate.

The cellular mechanism by which glucose stimulates the release of insulin from the pancreatic islets remains unresolved. Two main hypotheses have been put forth over the years to address this important question: (1) glucose interacts with a specific glucose 'receptor', thus setting into motion a cascade of events leading to insulin secretion; (2) initiation of insulin secretion occurs solely as a consequence of the metabolism of glucose (Hedekov, 1980). In support of the first hypothesis, it may be argued that glucose produces virtually instantaneous changes in the electrical activity of individual β -cells (Dean & Matthews, 1968; Atwater *et al.*, 1978; Beigelman & Ribalet, 1980), that may be dissociated from glucose metabolism (Pace & Tarvin, 1981), and similar rapid changes in insulin output measured from perfused islets. The effect of glucose on insulin secretion also demonstrates anomeric specificity (Rossini *et al.*, 1974; Grodsky *et al.*, 1975), with α -D-glucose being more effective than β -D-glucose, and moreover, compounds with similar molecular structures to glucose may also effect insulin release (Hellman *et al.*, 1972; Weaver *et al.*, 1979). The second hypo-

thesis is supported by evidence indicating that interference with the metabolism of glucose inhibits the ability of glucose to stimulate secretion (Malaisse *et al.*, 1968; Ashcroft *et al.*, 1970). Various investigators have alternatively argued that the explanation for the anomeric specificity of D-glucose in stimulating insulin secretion may be explained by the anomeric preference of several glycolytic enzymes involved in the metabolism of this hexose (Malaisse *et al.*, 1976; Meglasson & Matschinsky, 1983). Furthermore, no direct effect of glucose on an islet cellular process that may be related to insulin secretion has been unequivocally demonstrated.

We investigated the effect of the β -cell cytotoxin alloxan on a Ca^{2+} - and calmodulin-dependent protein kinase activity present in islet-cell homogenates and subcellular fractions (Colca *et al.*, 1983a). During the course of those studies we discovered that the presence of D-glucose in islet-cell homogenates unexpectedly resulted in the appearance of a 68 kDa phosphoprotein. As this was a specific effect of glucose, showed anomeric specificity, and was not shared by glucose 6-phosphate, the initial

supposition was that glucose was activating a protein kinase activity. However, a more detailed investigation demonstrated that the labelled 68 kDa phosphoprotein was in fact the phosphoryl intermediate of phosphoglucomutase. The data presented herein summarize these studies and describe the formation of this intermediate of phosphoglucomutase in the pancreatic islets. The possible significance of these findings with regard to glucose-stimulated insulin secretion is also discussed.

Experimental

Materials

Sprague-Dawley male rats (180–200 g) were obtained from Sasco (O'Fallon, MO, U.S.A.). Collagenase (type IV) was obtained from Worthington Diagnostic Systems, [γ - 32 P]ATP from New England Nuclear, and all other chemicals were purchased from Sigma Chemical Co. Glucose was also obtained from Fisher Scientific, Baker Chemical Co. and the National Bureau of Standards.

Preparation of tissues

Islets were prepared by collagenase digestion (Lacy & Kostianovsky, 1967) and purified on Ficoll gradients (Shibata *et al.*, 1976). Freshly isolated islets (1400–2500) from six to eight rats were extensively washed in fractionation buffer [50 mM-Mes (4-morpholine-ethanesulphonic acid)/250 mM-sucrose/1 mM-EDTA, pH 7.2] before homogenization in 650 μ l of fractionation buffer in a Kontes (0019) tissue homogenizer.

Subcellular fractionation of the islet homogenate was performed by a modification of the technique of Naber *et al.* (1980) as previously described (Colca *et al.*, 1982). Islets for subcellular fractionation were obtained from 30–33 rats and cultured overnight in CMRL 1066 media at 24°C (Colca *et al.*, 1982).

Adipocytes from rat epididymal fat-pads were prepared by a modification of the method of Rodbell (1964). Isolated adipocytes were washed in fractionation buffer before homogenization. The homogenate was removed from below the fat-cake. Liver and cerebral hemispheres of brain from rats were minced with scissors, washed extensively in fractionation buffer, and homogenized in a Teflon/glass Kontes (0019) homogenizer. Protein was measured with Fluorescamine (Orr *et al.*, 1976), with bovine serum albumin as standard.

Protein-phosphorylation assays

Protein-phosphorylation studies were performed as previously described for measuring Ca^{2+} - and calmodulin-dependent protein kinase activity (Colca *et al.*, 1983a). Briefly, protein-phos-

phorylation assays used tissue homogenates (10–50 μ g of protein/tube) or islet-cell subcellular fractions (10–25 μ g of protein/tube). The assays (50 μ l) were conducted at 37°C, pH 7.0, in 50 mM-Pipes (1,4-piperazinediethanesulphonic acid)/10 mM-MgCl₂/0.2 mM-EGTA, with or without 0.5 mM-CaCl₂. The reactions were initiated by the addition of 10 μ M-[γ - 32 P]ATP (5–10 μ Ci/tube) and terminated at the indicated times by the addition of sodium dodecyl sulphate (3%, w/v), 2-mercaptoethanol (2%, w/v) and glycerol (5%, v/v).

Unless indicated otherwise, hexose additions were made 5–10 s immediately before the initiation of the assays by [γ - 32 P]ATP. Glucose concentrations were standardized by measurement with a Beckman glucose analyser. For studies involving the anomers of D-glucose, the purified (95%) hexose crystals were dissolved in stock solutions 20 s before use. The respective glucose stocks were then allowed to mutarotate after completion of the protein kinase assay, so that the concentration of the stocks could be confirmed with the glucose analyser.

Phosphoproteins were separated on 10%-polyacrylamide slab gels at pH 8.6 for 6–16 h, as previously described (Landt *et al.*, 1982; Colca *et al.*, 1983b). The gels were stained with Coomassie Blue, destained, and exposed to X-ray film for 1–2 days. The phosphoprotein bands of interest were cut from the gel, by using the exposed film as a guide, hydrated, and dissolved in Protosol. 32 P incorporation was then quantified by liquid-scintillation counting. Glucose-dependent phosphorylation was defined as the phosphorylation that occurred in the presence of added glucose minus phosphorylation determined in the absence of glucose. Unless otherwise stated, *n* refers to the number of tissue preparations used for a given protocol.

Results

Effect of glucose on protein phosphorylation

In initial experiments, preincubation of islet-cell homogenates with 20 mM- or 50 mM-glucose for 5 min caused the appearance of a 68 kDa phosphoprotein when these assay mixtures were subsequently exposed to [γ - 32 P]ATP for 5 s. Similar results were obtained in the absence or the presence of Ca^{2+} (300 μ M); therefore Ca^{2+} was omitted from the subsequent assays. Quantitatively similar results were also obtained upon immediate addition of D-glucose (5–10 s) before initiation of the phosphorylation assay with omission of the preincubation period.

To determine if this protein phosphorylation was a specific and direct effect of glucose, similar experiments were undertaken with other hexoses

and hexose phosphates. L-Glucose, D-fructose and D-glucose 6-phosphate were ineffective (Fig. 1), as were 3-O-methyl-D-glucose, 2-deoxy-D-glucose, D-

Fig. 1. Specificity of glucose-dependent phosphorylation in islet-cell homogenates

Protein kinase assays were performed as described in the Experimental section. The reactions (5s) contained the indicated hexoses (5mM). The sucrose stock solution used for the incubation shown in line 6 was quantitatively converted into the invert sugars by a 15 min incubation with invertase before addition to the assay (lane 7) as described in the text. Assays shown in lanes 9 and 10 used the purified α - and β -anomers of D-glucose (0.85mM) as described in the text.

mannose and D-fructose 6-phosphate (5–20mM, $n = 3$) (results not shown). Whereas sucrose was inactive, quantitative conversion of sucrose into fructose and glucose by incubation with invertase (400 units) for 15 min at 55°C generated the active hexose (Fig. 1). Furthermore, the glucose-activated phosphorylation demonstrated an exquisite requirement for the α -anomer of D-glucose (Fig. 1). Minimal phosphorylation occurred in the presence of β -D-glucose, and may be attributed to contamination with the α -anomer.

Glucose-stimulated phosphorylation of the 68kDa protein band during a 5s assay was half-maximal at 0.34 ± 0.10 mM-glucose (S.E.M.; $n = 3$), and the rate of the reaction was maximal under these conditions at 5–10mM-glucose (Fig. 2). The rate of glucose-stimulated phosphorylation was linear for 10s, and dephosphorylation of the 68kDa phosphoprotein was evident only after 5–10min (Fig. 3). At short time periods there was little phosphorylation of the 68kDa band in the absence of exogenously added D-glucose. At longer incubation times (> 15 s) the 68kDa band became phosphorylated to a small degree even in the absence of added glucose.

The initial rate of glucose-dependent phosphorylation of the 68kDa protein was maximal between 10 and 20 μ M-ATP. This process also demonstrated an absolute requirement for Mg^{2+} . Under standard assay conditions this requirement was saturated at 1mM-MgCl₂ ($n = 3$; results not shown).

Fig. 2. Dependence of glucose-stimulated phosphorylation on the concentration of glucose

Protein kinase assays (5s) with islet-cell homogenates were performed as described in the Experimental section at the indicated concentrations of glucose. Fig. 2(a) is an autoradiogram and Fig. 2(b) shows the glucose-activated phosphorylation of the 68kDa protein for a representative experiment (mean $\pm$ S.E.M., $n = 3$). Phosphorylation determined in the absence of glucose was 0.26 ± 0.13 pmol/mg of protein and was subtracted from the data points shown.

Fig. 3. Time course of glucose-stimulated phosphorylation in islet-cell homogenates

Protein kinase assays were performed as described in the Experimental section with and without 5 mM-D-glucose. Data are means $\pm$ S.E.M. ($n = 3$) for glucose-activated phosphorylation of the 68 kDa protein band.

Mannoheptulose, an inhibitor of glucose phosphorylation which inhibits insulin secretion (Zawalich *et al.*, 1977), inhibited the glucose-stimulated protein phosphorylation. At 0.5 mM-glucose, 7 mM-mannoheptulose produced a 50% inhibition of the glucose-stimulated phosphorylation of the endogenous islet-cell protein. The inhibition was competitive with respect to glucose. Thus in three experiments the half-maximal activation of phosphorylation (10s incubations) occurred at 0.3 ± 0.1 mM-, 2.1 ± 1.3 mM- and 3.5 ± 0.1 mM-glucose (means $\pm$ S.E.M.) in the presence of 0, 10 mM- and 20 mM-mannoheptulose respectively, while the maximum velocity remained unchanged.

Experiments were undertaken to determine if this glucose-stimulated phosphorylation was unique to the pancreatic islets or was present also in other tissues. Protein-phosphorylation assays were carried out by the standard technique (5s) with and without 5 mM-D-glucose. Glucose-stimulated phosphorylation of the 68 kDa protein was shown in homogenates of islets, fat and cerebral hemispheres of brain, but was not demonstrated under these conditions in homogenates of liver ($n = 4$; results not shown). Glucose-stimulated phosphorylation was, however, subsequently demonstrated in liver homogenates incubated for 30s or more.

Experiments were performed to determine the subcellular location of the glucose-activated phos-

phorylation in islets. Protein-phosphorylation determinations (10s) in the presence and absence of 5 mM-glucose were made on islet-cell subcellular fractions enriched in plasma membrane, secretory granules and mitochondria, and in a microsomal fraction enriched in endoplasmic reticulum. The glucose-activated protein phosphorylation activity was absent from all three membrane fractions, but was present in the islet-cell cytosol. These results suggested that at least one component of the activity was missing from the membrane fractions. The addition of cytosol to fractions enriched in secretory granules or plasma membrane produced a synergistic increase in glucose-stimulated protein phosphorylation (10s assays; results not shown). In four separate experiments, this effect of cytosol on the membrane fractions was completely abolished by prior heat treatment of the cytosol for 10 min at 100°C. To determine if the component of the activity present in the membrane fractions was the 68 kDa protein substrate, glucose-activated protein-phosphorylation assays were performed in the combined presence of the secretory-granule fraction and cytosol for 1 min. The membranes were then rapidly sedimented (100000g for 10 min), and the resulting pellet and supernatant were subjected to the standard sodium dodecyl sulphate/polyacrylamide-gel-electrophoretic separation. The radioactive 68 kDa protein substrate was quantitatively recovered in the supernatant. The same result was obtained after centrifugal and electrophoretic separation of protein-phosphorylation assays that used the whole islet homogenate; i.e. the phosphorylated 68 kDa protein was recovered quantitatively in the cytosol, with no measurable 68 kDa phosphoprotein in the 100000g pellet. The results indicated that the protein phosphorylated in the presence of glucose is a cytosolic protein, and membranes contain an activator of this process.

Nature of the glucose-stimulated phosphorylation

Although the time course of the glucose-stimulated phosphorylation of the 68 kDa protein is suggestive of a protein kinase activity, it is conceivable that this effect of glucose could result from inhibition of a phosphatase activity or activation of a proteinase. To address the former possibility, protein kinase assays were carried out in the presence or absence of NaF (10 mM), a general inhibitor of protein phosphatases (Kasai & Field, 1983). Indeed, 10 mM-NaF decreased the dephosphorylation of the 68 kDa phosphoprotein that occurred in 5 and 10 min incubations (Table 1). However, the addition of 10 mM-NaF did not decrease the rate of glucose-stimulated phosphorylation of the 68 kDa protein (Table 1) in 1 min assays. These results indicate that this effect of glucose is not due to inhibition of a phosphoprotein phosphatase.

Table 1. Effect of fluoride on glucose-stimulated phosphorylation

Protein kinase assays utilizing islet-cell homogenates were incubated for the indicated times with or without fluoride (10mM). Phosphorylation of the 68 kDa protein was determined as described in the text. Data are means $\pm$ S.E.M. ($n = 3$) and represent phosphorylation in the presence of 5mM-glucose minus that determined in the absence of glucose.

	Incubation time (min) ...	Glucose-dependent P _i incorporation (pmol/mg of protein)		
		1	5	10
Control		1.58 $\pm$ 0.44	0.87 $\pm$ 0.25	0.58 $\pm$ 0.18
Fluoride (10mM)		1.65 $\pm$ 0.44	1.38 $\pm$ 0.43	1.06 $\pm$ 0.27

To rule out the possibility that the 68 kDa phosphoprotein appeared as a result of a glucose-activated proteolysis, protein phosphorylation was allowed to proceed in the presence of 10 μ M- $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ for 5 min at 37°C in the absence of added glucose. After this 5 min incubation, the reaction tubes received 10 μ M unlabelled ATP, with or without 5mM-glucose. The addition of glucose under these conditions did not alter the phosphorylation of the 68 kDa band and indicated that the effect of glucose required the simultaneous presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (results not shown). The possibility that this phosphoprotein band may represent a catalytic intermediate involving ATP was also addressed. Glucose-activated phosphorylation was allowed to proceed for 5 min, and then increasing concentrations of unlabelled ATP were added to the incubation medium in an attempt to replace the ^{32}P radiolabel. Under these conditions, the ^{32}P radiolabel could not be chased from the 68 kDa protein band by ATP (results not shown). Furthermore, the addition of hexokinase, glucose and ATP in the complete assay mixture in the absence of membranes did not form a catalytic intermediate detectable after electrophoretic separation by these techniques.

Although the addition of glucose 6-phosphate together with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ under the standard assay conditions did not label the 68 kDa islet-cell protein, the possibility existed that glucose 6-phosphate may affect the phosphorylation of the protein in the presence of glucose. Indeed, in the presence of 50 μ M-D-glucose 6-phosphate, glucose-stimulated labelling of the protein did not occur, even in the presence of 20mM-D-glucose (results not shown). Further studies demonstrated that the addition of glucose 6-phosphate (1–50 μ M) to the pre-phosphorylated islet-cell protein resulted in the rapid removal of ^{32}P . This dephosphorylation occurred very rapidly and was complete within 5 s whether the addition of the glucose 6-phosphate was at 37 or 4°C. The nature of this ‘chase’ of the radioactive label was suggestive of a catalytic turnover. Thus the ability of several hexose phosphates to chase the label from the 68 kDa protein was

Fig. 4. Ability of various hexose phosphates to dephosphorylate the ^{32}P -labelled 68 kDa islet-cell protein. Islet-cell homogenates were incubated with $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$, with or without 5mM-glucose, for 1 min as described in the Experimental section. The ability of various hexose phosphates (50 μ M) to dephosphorylate the protein was assessed in an additional 5 s incubation. Samples were then separated on electrophoretic gels. The autoradiogram (origin at top) is representative of three experiments. Abbreviations: Glc6P, glucose 6-phosphate; Glc1,6P₂, glucose 1,6-bisphosphate; Fru6P, fructose 6-phosphate; Fru1P, fructose 1-phosphate; Fru1,6P₂, fructose 1,6-bisphosphate; Fru2,6P₂, fructose 2,6-bisphosphate; 6PGlcA, 6-phosphogluconate.

evaluated. At 50 μ M-hexose phosphate, the ^{32}P was effectively chased from the islet-cell protein by α -D-glucose 1-phosphate, D-glucose 6-phosphate and D-fructose 6-phosphate. A minor effect was also observed with glucose 1,6-bisphosphate; however, under these conditions, β -D-glucose 1-phosphate, D-fructose 1-phosphate, D-fructose 1,6-bisphosphate, D-fructose 2,6-bisphosphate and 6-phosphogluconate were ineffective (Fig. 4). This pattern of dephosphorylation by these hexose phosphates suggested that the islet-cell protein may be phosphoglucomutase, which is a phosphoenzyme in its active catalytic state (Jagannathan & Luck, 1949; Ray & Gertrude, 1964; Britton & Clarke, 1968). Indeed, when 5 μ g of purified skeletal-muscle

Fig. 5. Phosphorylation of the 68 kDa islet-cell protein and phosphoglucosmutase

Islet-cell cytosol (10 μ g of protein) or purified phosphoglucosmutase (3 μ g) was incubated with glucose 6- 32 P]phosphate for 1 min in the presence or absence of 20 mM-mannoheptulose (MH). After this incubation, the ability of various hexose phosphates (50 μ M) to 'chase' the 32 P label was assessed in an additional 5 s incubation (abbreviations: 3PG, 3-phosphoglyceric acid; others as in Fig. 4). The autoradiogram is representative of results obtained in three experiments.

Table 2. Effect of cyclic AMP-dependent protein kinase on 32 P incorporation into phosphoglucosmutase

Purified phosphoglucosmutase was incubated for 60 min with or without (control) 4 units of the catalytic subunit of cyclic AMP-dependent protein kinase in the presence of 10 μ M- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as described in the text. The label was then chased from the phosphoglucosmutase by an additional 5 s incubation with 50 μ M-glucose 6-phosphate or α -glucose 1-phosphate. Results are means $\pm$ S.E.M. ($n = 3$).

	P _i incorporation (pmol/mg of protein)
Control	12.8 $\pm$ 0.70
+ Cyclic AMP-dependent protein kinase. Chase:	
None	85.4 $\pm$ 4.9
Glucose 6-phosphate	7.6 $\pm$ 3.1
α -Glucose 1-phosphate	5.4 $\pm$ 1.2

phosphoglucosmutase was added to the islet-cell homogenate in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, the enzyme was labelled in a glucose-activated manner, and the labelled skeletal-muscle enzyme was not resolved from the islet-cell protein on the electrophoretic gels. Furthermore, once both proteins were labelled, the label was chased by α -glucose 1-phosphate and glucose 6-phosphate.

To determine if glucose labelled the islet-cell protein and the added skeletal-muscle phosphoglucosmutase by generation of ^{32}P glucose 6-phosphate, labelled glucose 6-phosphate was generated by incubation (30 min) of glucose with yeast hexokinase and 10 μ M- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The addition of glucose 6- ^{32}P phosphate to islet-cell homogenates, islet-cell cytosol or the purified phosphoglucosmutase labelled the 68 kDa band in the islet homogenates as well as, in separate incubations, the purified

mutase. This labelling occurred in the absence of ATP and was not inhibited by 20 mM-mannoheptulose (Fig. 5). Furthermore, both the islet-cell protein and the purified phosphoglucosmutase, once labelled by incubation with glucose 6- ^{32}P phosphate, could be dephosphorylated in a similar manner, i.e. the label was chased by subsequent incubation with α -glucose 1-phosphate and glucose 6-phosphate.

Interestingly, when concentrated islet-cell or brain cytosol (20–30 μ g/assay) was exposed to glucose 6- ^{32}P phosphate under these assay conditions, two phosphoproteins other than the phosphoglucosmutase were labelled. One band migrated with an apparent M_r of 70 000, and another at about 90 000. Glucose 6-phosphate and α -glucose 1-phosphate were also able to dephosphorylate these proteins (results not shown).

To determine if the islet-cell phosphoglucosmutase could be phosphorylated by another mechanism, the islet-cell cytosol or purified phosphoglucosmutase was incubated with and without 4 units of the catalytic subunit of cyclic AMP-dependent protein kinase and 10 μ M- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Phosphoglucosmutase was found to serve as a substrate for the kinase (Table 2). Furthermore, the ^{32}P incorporated in the presence of cyclic AMP-dependent protein kinase was chased from the mutase by α -glucose 1-phosphate and glucose 6-phosphate (Table 2). The addition of the catalytic subunit of cyclic AMP-dependent protein kinase increased the phosphorylation of numerous protein bands in the islet-cell cytosol. The increase in the background radioactivity made it impossible to discern whether the endogenous phosphoglucosmutase was affected even when unlabelled glucose 6-phosphate was added in an attempt to locate the

Table 3. *Mannoheptulose-insensitive phosphorylation of phosphoglucomutase (PGM) by islet-cell cytosol*

Phosphorylation assays with [γ - 32 P]ATP were performed for 30 min as indicated in the text. Incubations contained 20 mM-mannoheptulose and no glucose to ensure that phosphorylation of the PGM occurred independently of generation of radiolabelled hexose phosphates. Assays contained 3 μ g of purified PGM with or without islet-cell cytosol (12 μ g of protein/tube), or cytosol alone. For some incubations, heat-treated cytosol (100°C, 15 min) was added to the exogenous PGM. The synergistic effect of cytosol and PGM occurred unimpeded in the presence of 40 mM- or 80 mM-mannoheptulose under these conditions.

	Incorporation into PGM (c.p.m.)
PGM	662 $\pm$ 105
Cytosol	716 $\pm$ 121
Cytosol + PGM	12768 $\pm$ 2417
Heat-treated cytosol + PGM	1339 $\pm$ 66

mutase in the resulting autoradiograms (results not shown).

To analyse whether the islet-cell cytosol contained a kinase activity that could phosphorylate exogenous phosphoglucomutase, 3 μ g of purified phosphoglucomutase was incubated with or without islet-cell cytosol. Mannoheptulose (20, 40 or 80 mM) was added to prevent formation of labelled glucose 6-phosphate, which would confound the results. (Endogenous glucose measured fluorimetrically with a coupled enzyme assay was less than 20 μ M.) The islet-cell cytosol contained a heat-labile factor that phosphorylated exogenous phos-

by the islet-cell fractions is rate-limiting for demonstration of the radiolabelled enzyme under these conditions. Thus the specific requirements for α -D-glucose, ATP and Mg^{2+} for labelling the protein are necessary for activation of endogenous hexokinase activity. Since the hexokinase reaction is rate-limiting, addition of islet-cell membranes, which contain hexokinase (Matschinsky, 1972), augments the observed phosphorylation of the mutase. The labelling of the islet-cell phosphoglucomutase was also increased after addition of exogenous hexokinase, and inhibited by mannoheptulose, which is a competitive inhibitor of hexokinase. Furthermore, direct addition of glucose 6- 32 P]phosphate labelled the 68 kDa cytosolic protein as well as purified phosphoglucomutase in the absence of ATP; this process was not affected by mannoheptulose. Interestingly, fructose and mannose when added to the islet-cell homogenates in the presence of [γ - 32 P]ATP failed to label the phosphoglucomutase. Presumably, these results occur, on the one hand, owing to the poor ability of the islet-cell hexokinase to phosphorylate fructose, and on the other, as a result of the low activity of phosphomannoisomerase present in the pancreatic islets (Ashcroft & Randle, 1970; Matschinsky, 1972). Thus the addition of fructose phosphorylated by yeast hexokinase effectively labelled the mutase in islet-cell homogenates, whereas the addition of mannose 6- 32 P]phosphate had no effect (1 min incubations; results not shown).

The labelling of the islet-cell phosphoglucomutase (PGM) under these conditions occurs when radioactive (*) glucose 6-phosphate reacts with the endogenous phosphoenzyme, i.e.:

phoglucomutase (Table 3). This effect of the cytosol on phosphorylation of phosphoglucomutase continued at a linear rate for 30 min (results not shown) and was not affected by a 4-fold change in mannoheptulose concentration in the medium. The radioactive phosphate was chased from the phosphoglucomutase by glucose 6-phosphate and glucose 1-phosphate (results not shown).

Discussion

These studies describe a glucose-activated phosphorylation of a 68 kDa protein in islet-cell homogenates. The phosphorylated protein is islet-cell phosphoglucomutase, which is labelled as part of the catalytic cycle in the presence of glucose and ATP after the generation of glucose 6- 32 P]phosphate. The generation of glucose 6- 32 P]phosphate

Thus the radioactive phosphate can be chased from the enzyme by the subsequent addition of either glucose 1-phosphate or glucose 6-phosphate. The enzyme exhibits α -anomeric specificity; therefore β -glucose 1-phosphate is without effect. The ability of fructose 6-phosphate to chase the 32 P from the enzyme may result from a direct ability of fructose 6-phosphate to accept the phosphate from the enzyme, as shown by Passonneau *et al.* (1969), or may result after rapid conversion into glucose 6-phosphate by phosphoglucoisomerase. Fructose 6-phosphate was more effective in chasing the 32 P label from the endogenous islet phosphoglucomutase in the cell homogenates than from the purified phosphoglucomutase. As the purified phosphoglucomutase is undoubtedly contaminated with phosphoglucoisomerase, it is possible that this effect of fructose 6-phosphate may also be indirect.

Phosphoglucosmutase activity was only recently measured in the pancreatic islets by Malaisse and colleagues (Malaisse-Lagae *et al.*, 1982; Sener *et al.*, 1982). They suggested that phosphoglucosmutase may be responsible for the synthesis of a portion of glucose 1,6-bisphosphate in pancreatic islets, as had been originally proposed to occur in brain (Passonneau *et al.*, 1969). In fact, intact islets treated with D-glucose have augmented cytosolic concentrations of glucose 1,6-bisphosphate (Sener *et al.*, 1982). The current study substantiates the existence of phosphoglucosmutase in the pancreatic islets by demonstration of the phosphoenzyme in islet-cell cytosol after electrophoretic separation on slab gels.

The current study also corroborates the findings of Kirschenlohr & Hofer (1983) and Beitner & Cohen (1980), which demonstrated that phosphoglucosmutase can serve as a substrate for cyclic AMP-dependent protein kinase. This protein kinase seems to phosphorylate the phosphoglucosmutase at the catalytic site, since the incorporated phosphate is chased in the same manner as phosphate incorporated during the catalytic cycle. Once the enzyme is so phosphorylated, the addition of glucose 1-phosphate or glucose 6-phosphate as a phosphate acceptor then generates glucose 1,6-bisphosphate, a certain portion of which may be released from the central enzyme complex and contribute to the cellular content of free glucose 1,6-bisphosphate. Thus phosphorylation of phosphoglucosmutase by cyclic AMP-dependent protein kinase may be a cellular mechanism for generation of glucose 1,6-bisphosphate.

The importance of glucose 1,6-bisphosphate as a regulator of cellular metabolism appears to be widespread. Thus the bisphosphate is an activator of phosphofructokinase (Krzanowski & Matschinsky, 1969) and phosphoglucosmutase, and modulates the activity of hexokinase, pyruvate kinase and 6-phosphoglucosmutase dehydrogenase (Beitner *et al.*, 1975; Beitner, 1979). It is also possible that glucose 1,6-bisphosphate may serve as an important cellular signal for initiation and modulation of insulin secretion, via a direct effect of the bisphosphate or by regulation of the rate of glucose metabolism. Circumstantial evidence that would favour such a hypothesis would include the ability of α -D-glucose, the most important insulin secretagogue, to increase glucose 1,6-bisphosphate concentrations in islet homogenates (Malaisse-Lagae *et al.*, 1982; Sener *et al.*, 1982), as well as the pattern of phosphorylation of the mutase observed in the present study. It is also noted that the glucose bisphosphate concentrations in other tissues decrease with starvation and increase after protocols that raise cellular concentrations of cyclic AMP (Beitner *et al.*, 1979). These same protocols either

decrease or increase insulin secretion, respectively.

The cellular concentration of glucose 1,6-bisphosphate may be controlled by the activity of phosphoglucosmutase, perhaps involving cyclic AMP-dependent phosphorylation, but also by the action of glucose 1,6-bisphosphate synthase (Rose *et al.*, 1975; Wong & Rose, 1976) and glucose 1,6-bisphosphatase (Guha & Rose, 1982, 1983). It is important to note that the activity of glucose 1,6-bisphosphatase has been shown to increase with starvation and to be lowered with increased cellular cyclic AMP (Beitner & Cohen, 1979). Interestingly, both of these enzymes, glucose 1,6-bisphosphatase synthase and phosphatase, are theorized to form catalytic phospho-intermediates similar to that formed by phosphoglucosmutase. The M_r values of these enzymes are 70000 and 87000 respectively. The demonstration of phosphoproteins, in islet and brain, of similar size which are labelled by glucose 6- $[^{32}\text{P}]$ phosphate and chased by α -glucose 1-phosphate and glucose 6-phosphate suggests that these activities, which have been enzymically characterized in brain, may also exist in the pancreatic islets.

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