Protein Kinase A-dependent Phosphorylation of GLUT2 in Pancreatic β Cells

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In pancreatic β cells, cyclic AMP-dependent protein kinase regulates many cellular processes including the potentiation of insulin secretion. The substrates for this kinase, however, have not been biochemically characterized. Here we demonstrate that the glucose transporter GLUT2 is rapidly phosphorylated by protein kinase A following activation of adenyl cyclase by forskolin or the incretin hormone glucagon-like peptide-1. We show that serines 489 and 501/503 and threonine 510 in the carboxyl-terminal tail of the transporter are the in vitro and in vivo sites of phosphorylation. Stimulation of GLUT2 phosphorylation in β cells reduces the initial rate of 3-O-methyl glucose uptake by ~48%; but does not change the Michaelis constant. Similar differences in transport kinetics are observed when comparing the transport activity of GLUT2 mutants stably expressed in insulinoma cell lines and containing glutamates or alanines at the phosphorylation sites. These data indicate that phosphorylation of GLUT2 carboxy-terminal tail modifies the rate of transport. This lends further support for an important role of the transporter cytoplasmic tail in the modulation of catalytic activity. Finally, because activation of protein kinase A stimulates glucose-induced insulin secretion, we discuss the possible involvement of GLUT2 phosphorylation in the amplification of the glucose signaling process.

Basal intracellular cAMP levels are critically required to maintain the glucose-dependent secretory activity of pancreatic β cells. This is evidenced by the poor secretory response of cell sorter purified β cells, which have a low intracellular cAMP content. Activation of adenyl cyclase, however, restores a normal responsiveness to elevations in extracellular glucose (Pipeleers et al., 1985; Schuit and Pipeleers, 1985; Yada et al., 1993; Wang et al., 1993). Furthermore, acute increases in intracellular cAMP above basal levels can further strongly potentiate glucose-induced insulin secretion. This occurs for instance following nutrient ingestion when carbohydrates and fat induce the secretion by gut endocrine cells of two peptide hormones, GIP (glucose-dependent insulinohippertic polypeptide) and GLP-1 (glucagon-like peptide-1) (Creutzfeld and Nauck, 1992; Dupre, 1991). These hormones bind to specific β cell G protein-coupled receptors that activate the adenyl cyclase pathway and strongly potentiate glucose-induced insulin secretion (Thorens, 1992a, 1994; Usdin et al., 1993; Widmann et al., 1994; Gremlich et al., 1995). The role of CAMP on the glucose competence state of β cells and its acute stimulatory effects on insulin secretion indicate that multiple protein kinase A-dependent phosphorylation events are involved in the modulation of the insulin secretory activity. The substrates for PKA in β cells, however, have not been biochemically characterized.

The glucose transporter GLUT2 belongs to the family of facilitated diffusion hexose transporters and is specifically expressed in tissues that carry large glucose fluxes such as intestine, liver, the proximal part of the kidney nephron, and pancreatic β cells (Thorens, 1992b). In β cells, GLUT2 catalyzes the uptake of glucose, which is the first step in glucose-induced insulin secretion. Glucose is then phosphorylated by glucokinase and following its metabolism it induces insulin secretion by triggering plasma membrane depolarization, influx of calcium, and fusion of secretory granules with the plasma membrane. In the normal physiological state, the rate-limiting step in this process is the phosphorylation of glucose by glucokinase (Meglasson and Matschinsky, 1986).

Recent evidence has suggested, however, that GLUT2 may participate in the glucose signaling process by a mechanism distinct from its role as a sugar transporter. For instance, Newgard and collaborators stably transfected insulinoma cell lines that had lost GLUT2 expression and glucose-induced insulin secretion with GLUT2 or GLUT1. Only the GLUT2-expressing cells and not the GLUT1-expressing cells recovered a glucose-dependent secretory response, although the rate of glucose metabolism was identical in both transfectedants. Furthermore, when the effect of forskolin was tested on the secretory activity of the transfected cells, the increment in insulin secretion was higher in the GLUT2-expressing cells (Hughes et al., 1992, 1993; Ferber et al., 1994). Also, Valera et al. (1994) observed that a ~70–80% reduction in GLUT2 expression in β cells of transgenic mice expressing GLUT2 antisense RNA leads to a decreased insulin secretory response and to diabetes. This suggests that even in conditions in which transport activity should not be limiting for glucose metabolism, β cell diabetic dysfunctions develop. This therefore indicates that GLUT2 may serve a signaling role in β cells distinct from its transport activity.
Materials and Methods

Cells and Cell Culture—RINm5F cells were kept in culture in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. Stable transfection of insulinoma with expression plasmids was by electroporation. Briefly, cells were trypsinized, washed in phosphate-buffered saline, and resuspended at 10⁶ in 0.8 ml of PBS (8 mM Na₂HPO₄, 2H₂O, 137 mM NaCl, 1.5 mM KH₂PO₄, 15 mM KCl, 2.7 mM CaCl₂, 0.1% Triton X-100) containing 1% of the plasmid. Electroporation was performed at 500 microfarad and 300 V; the cells were then kept for 10 min in the electroporation cuvette and transferred to 40 ml of prewarmed medium before being plated in tissue culture dishes. Selection with G418 at 400 μg/ml was started 2–3 days later. Pancreatic islets were isolated by collagenase digestion of pancreas and were then resuspended in the same medium and plated on gelatin-coated dishes (Gotoh et al., 1987). β cells were purified from dispersed islets by autofluorescence-activated cell sorting (Pipeleers and Van De Winkel, 1989; Giordano et al., 1993) and kept overnight in RPMI 1640 medium containing 10% fetal calf serum before phosphate labeling experiments.

Phosphoamino Acid Analysis—Phosphoamino acid analysis was performed using the method of Eancer and Long (1989). All the constructions were verified by sequence analysis.

Transport Assays—For measurement of 3-O-methyl glucose uptake kinetics, cells were plated in 6-well plates and used when confluent. Before initiation of the assays, cells were washed twice with PBS, incubated in 2 ml of Krebs-Ringer bicarbonate buffer containing 10 mM Hepes, pH 7.4, and 0.5% BSA (KRHB-BSA) two times for 30 min at 37°C, washed once with PBS, and maintained in KRHB-BSA until used for the uptake. Uptake was initiated by aspirating the medium and adding 1 ml of 3-O-methyl-[1-¹⁴C]-glucose at concentrations ranging from 1 to 50 μM for uptake experiments with transfected cells and from 0.1 to 10 mM for untransfected cells. Radioactive tracer was present at 5–10 μCi/ml. Uptake were performed at 19–20°C and stopped after periods ranging from 10 to 60 s by the rapid addition of 2 ml of ice-cold PBS containing 1 mM HgCl₂ and five additional washings with the same solution. The cells were lysed in 1% SDS; an aliquot of the lysate was electrophoresed on SDS-7.5% polyacrylamide gels, and the gel was stained with Coomassie blue and dried. The autoradiographs were quantified by densitometry.

RESULTS

The carboxy-terminal cytoplasmic tail of GLUT2 contains several potential sites for phosphorylation by protein kinase A (Fig. 1)(Fukumoto et al., 1988; Thorens et al., 1988; Suzue et al., 1989). Except for threonine at position 510, which is unique to the rat GLUT2 sequence, the other PKA sites are also present in mouse and human GLUT2.

To determine whether GLUT2 present in β cells was phosphorylated in response to elevations in intracellular cAMP, we...
labeled pancreatic islets with radioactive phosphate and exposed them to CPT-cAMP and IBMX for different periods of time. GLUT2 was then immunoprecipitated from cell lysates and analyzed by gel electrophoresis (Fig. 2). A basal level of GLUT2 phosphorylation was observed in the absence of stimuli but was not increased by the drug treatment for periods from 1 to 10 min. Similar observations were made when forskolin or dibutyryl cAMP were used instead of CPT-cAMP (not shown). The inability to increase the phosphorylation of GLUT2 in intact islets may be due to the presence of glucagon secreting cells. Indeed, β cells have receptors for glucagon that are coupled to activation of adenyl cyclase (Van Schravendick et al., 1985; Jelinek et al., 1993; Abrahamsen and Nishimura, 1995) and that may therefore maintain a high intracellular cAMP level (Schult and Pipeleers, 1985), thus preventing further stimulation of PKA-dependent phosphorylation. If this were the case, phosphorylation experiments performed on cell sorter purified β cells should allow us to detect the stimulatory effect of increases in cAMP on GLUT2 phosphorylation. Indeed, when experiments were carried out with purified β cells, there was a low basal level of GLUT2 phosphorylation that could however be stimulated by the addition of forskolin or GLP-1 (Fig. 3A). The quantification and summary of the data obtained in four different experiments with purified β cells is presented in Table I and shows that GLP-1 was as potent as forskolin in inducing GLUT2 phosphorylation and that maximal phosphorylation was already obtained 5 min after the addition of the stimuli.

To determine whether PKA was responsible for the observed phosphorylation, an experiment was carried out in the presence or the absence of the PKA inhibitor H-89 (Chijiiwa et al., 1990). As shown in Fig. 3B, the presence of the inhibitor completely prevented the stimulation of GLUT2 phosphorylation. The phosphorylated amino acids present in GLUT2 were then determined by phosphoamino acid analysis. As shown in Fig. 3C, both phosphoserines and phosphothreonines were detected.

As a first approach to identify the phosphorylation sites, we prepared fusion proteins consisting of the glutathione S-transferase (GST) and either the middle intracellular loop (amino acids 237–301, GST-ML), which does not contain putative PKA sites, or the carboxy-terminal cytoplasmic tail (amino acids 481–522, GST-CT) of GLUT2. These proteins were phosphorylated in an in vitro reaction in the presence of the catalytic subunit of protein kinase A and radioactive ATP. In Fig. 4A, the left panel shows the Coomassie Blue staining of the fusion proteins from the in vitro reaction. On the right panel, the autoradiogram of the same gel demonstrates that only the fusion protein containing the carboxyl-terminal region (GST-CT) was phosphorylated by PKA, consistent with the presence of putative PKA sites in the transporter carboxyl-terminal tail. By phosphoamino acid analysis, both phosphoserines and phosphothreonines were detected in the fusion protein (Fig. 4C).

To define the phosphorylation sites, we generated four different variants of the GST-CT fusion proteins with various combination of serine and threonine mutations, as described in Fig. 1. Fig. 4B shows in the upper panel the Coomassie Blue staining of the fusion proteins and in the lower panel two different autoradiographic exposures of the same gel. In Fig. 4C, the phosphoamino acid maps of these fusion proteins are presented. From these experiments one can deduce that Thr<sup>510</sup> is the only threonine phosphorylated because its mutation led to the disappearance of phosphothreonine (Fig. 4C). With regards to serines, mutation of Ser<sup>501</sup>/<sup>503</sup> led to a fusion protein that was phosphorylated mostly on Thr but also, at a low level, on serines as can be detected after longer exposure of the thin layer chromatogram. Also, the GST-CT-SST-E fusion protein

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**Table I**

| Experiment | Activator | Time (min) | Phosphorylation (%) of control |
|------------|-----------|------------|-------------------------------|
| I          | Forskolin | 10         | 1020                          |
| II         | GLP-1     | 5          | 803                           |
| III        | Forskolin | 5          | 509                           |
|            | GLP-1     | 20         | 411                           |
| IV         | GLP-1     | 5          | 683                           |

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**Fig. 2.** GLUT2 phosphorylation in intact pancreatic islets. Pancreatic islets were labeled with radioactive phosphate for 2.5 h, and CPT-cAMP (1 mM) and IBMX (1 mM) were added for the indicated periods of time (minutes). After cell lysis, GLUT2 was immunoprecipitated and separated by gel electrophoresis on 7.5% SDS-polyacrylamide gels. In the absence of stimulation (time = 0) a basal level of GLUT2 phosphorylation was detected that was, however, not increased by the addition of CPT-cAMP and IBMX.

**Fig. 3.** A, GLUT2 phosphorylation in cell sorter purified β cells. β cells (5 × 10<sup>5</sup>) were labeled with radioactive phosphate for 2.5 h, and forskolin (FSK) at 100 μM or GLP-1 (10 nM) was added for the indicated periods of time in the presence of IBMX. After cell lysis, GLUT2 was immunoprecipitated and analyzed as in Fig. 2. A 5–10-fold stimulation of phosphorylation was detectable (see Table I). B, GLUT2 phosphorylation induced by GLP-1 in cell sorter purified β cells is prevented by the protein kinase A inhibitor H-89. β cells (5 × 10<sup>5</sup>) were labeled with radioactive phosphate for 2.5 h, and the inhibitor H-89 at 10 μM was added to the cells for the last hour of the radioactive labeling. Cells were then stimulated or not by GLP-1 in the presence of IBMX for 5 min. GLUT2 was immunoprecipitated and analyzed as described in the legend to Fig. 2. C, analysis of the phosphoamino acids from GLUT2 immunoprecipitated from the β cell lysates. Phosphoserines and phosphothreonines are detected.
with mutation of Ser501,503 and Thr510 was still phosphorylated on serines (Fig. 4C). Additional mutations of Ser489 (GST-CT-SSST-E) led to a protein that could no longer be phosphorylated. Thus, Ser489, Ser501,503, and Thr510 are the in vitro sites of PKA phosphorylation on the GLUT2 cytoplasmic tail.

To determine whether the phosphorylation sites identified with the fusion proteins were indeed those used in vivo, we transiently transfected Cos cells with wild-type GLUT2 or the SSST-E mutant. The cells were then labeled with radioactive orthophosphate and stimulated or not with forskolin in the presence of IBMX. As shown in Fig. 5, activation of adenyl cyclase led to an increase in GLUT2 phosphorylation but not in the phosphorylation of the SSST-E mutant. These data thus indicate that the phosphorylation sites identified in vitro on fusion proteins are indeed used in intact cells by activated protein kinase A. Also, because a basal level of phosphorylation is observed in the absence of stimulation of adenyly cyclase in both the wild-type and mutant forms, this indicates that there are one or more additional sites in GLUT2 that are phosphorylated by an as yet not identified kinase.

To determine whether phosphorylation of GLUT2 in normal β cells induced a change in the kinetics of glucose uptake, cell sorter purified β cells were stimulated or not with GLP-1 in the presence of IBMX in conditions that give maximal phosphorylation of GLUT2. The cells were then used to determine the initial uptake velocity in the presence of 20 mM 3-O-methyl glucose. As shown in Fig. 6, pretreatment of the cells with GLP-1 led to a reduction in the initial velocity by about 40%. Measurement of 3-O-methyl glucose uptake over a range of concentrations (Fig. 6B) further indicated that in the absence of GLP-1, the Km value for uptake (14.7 mM) was not significantly different from that reported in preceding publications (17–18 mM) (Johnson et al., 1990; Heimberg et al., 1993, 1995). These data thus indicate a decrease in the catalytic activity of the transporter upon phosphorylation.

We next assessed whether the presence of negative charges at the sites of phosphorylation could result in a change of transport kinetics similar to that observed with intact β cells stimulated with GLP-1. We thus constructed GLUT2 mutants in which the identified phosphorylation sites were mutated either to alanine (GLUT2 SSST-A) or to glutamate (GLUT2 SSST-E). In these mutants, no PKA-dependent phosphorylation can take place, and in the SSST-E mutant, the glutamate residues introduce negative charges that may mimic the phosphorylated state of GLUT2, as reported for the L-type calcium channel for instance (Li et al., 1993). Both transporter mutants were stably transfected in RINm5F cells. Selected clones were then used in glucose uptake experiments to determine Km and Vmax. Total expression of GLUT2 in these cell lines was determined by Western blot analysis, and surface expression of the transporter was verified by a trypsin assay performed at 4°C (Thorens et al., 1993). Fig. 7A shows by quantitative Western

Fig. 4. In vitro phosphorylation of fusion proteins by the catalytic subunit of protein kinase A. Fusion proteins consisting of the GST protein and different segments of wild-type or mutated GLUT2 were incubated in the presence of PKA and radioactive ATP. Incorporation of radioactivity was determined after separation of the reaction mixture by gel electrophoresis and autoradiography. A, on the left is the Coomassie Blue staining of the different proteins: GST, GST-CT (CT indicates the cytoplasmic tail of GLUT2), and GST-ML (ML indicates the middle loop of GLUT2). The lower bands in the last two lanes probably represent prematurely stopped translation products. On the right is the autoradiogram of the same gel. Only the band corresponding to the fusion protein containing the cytoplasmic tail of GLUT2 was labeled. B, in vitro phosphorylation of GST fusion proteins containing mutated forms of the cytoplasmic tail of GLUT2 (see the legend to Fig. 1 for a description). The upper panel is the Coomassie Blue staining of the gel and indicates that equal amounts of proteins were used in the phosphorylation reactions. Lower panels, short and long autoradiographic exposures of the Coomassie Blue-stained gel.

Fig. 5. Phosphorylation of GLUT2 and the SSST-E mutant transiently transfected in Cos cells. Transfected Cos cells were labeled with [32P]orthophosphate and accumulation of intracellular cAMP and activation of PKA were stimulated by the addition of forskolin and IBMX for 15 min. The wild-type and mutated transporters were then immunoprecipitated and separated on polyacrylamide gels. Densitometry scanning analysis shows a 4.8-fold stimulation of wild-type GLUT2 phosphorylation. This is a representative experiment out of three performed.
Glut2 Phosphorylation in β Cells

Identified phosphorylation sites in COS cells. Only wild-type GLUT2 phosphorylation can be increased upon stimulation of adenylyl cyclase with forskolin. In these experiments, however, the SSST-E mutant was still phosphorylated to a low basal level, indicating that the transporter is phosphorylated on an additional site by a kinase different from protein kinase A that has not yet been identified.

3-O-Methyl glucose uptake experiments performed on cell sorter purified β cells in the presence or the absence of GLP-1 indicated that under conditions that induce maximal phosphorylation of GLUT2, there was a −48% reduction in the initial uptake velocity measured in the presence of 20 mM substrate. This concentration of 3-O-methyl glucose is slightly above the Km of the transporter. These experiments are difficult to perform at higher substrate concentrations because the number of cells is a limiting factor in these experiments and with more diluted radioactive tracers the accuracy of the data decreases. Nevertheless, these data provide indications that a consequence of GLUT2 phosphorylation is a reduction of its catalytic activity. Furthermore, we know from previous experiments (Thorens et al., 1993) that GLUT2 is permanently expressed at the cell surface even in intact islets in which the basal level of phosphorylation is elevated. Thus stimulation of GLUT2 phosphorylation is not accompanied by internalization of the transporter, and decrease in initial uptake rate cannot be accounted for by a decreased cell surface expression of the transporter.

Uptake experiments conducted with mutated transporters expressed in insulinoma cells not expressing the endogenous GLUT2, indicated that the mutant with glutamate residues in place of serines 489 and 501/503 and threonine 510 had a Vmax reduced by −40% compared with the alanine mutant. These data very closely correlate with the observed effect of phosphorylation of GLUT2 in β cells and strongly suggest that introduction of negative charges by substitution of serines and threonines by the acidic amino acids glutamate mimics the phosphorylated state of the transporter. Similar conclusions have also been drawn, for instance, in the case of the L-type Ca2+ channel (Li et al., 1993).

When glucose uptake is measured using dispersed islet cells, two transport components are observed with values of −17 mW and 1–2 mW (Johnson et al., 1990; Heimberg et al., 1993). In purified β cells, only the high Km component is observed (Heimberg et al., 1995). Because in purified β cells GLUT2 is only very poorly phosphorylated, whereas in dispersed islets cells that contain glucagon cells its level of phosphorylation may be higher, we hypothesized that the phosphorylated form of GLUT2 may account for the presence of the low Km transport component. However, this is not supported by the present experimental evidences. Indeed, the Km for 3-O-methyl glucose uptake measured upon stimulation of β cells with GLP-1 had a value not very different from values published previously (14.8 mW versus 17–18 mW) (Johnson et al., 1990; Heimberg et al., 1993, 1995). Experiments performed with the glutamate mutant expressed in insulinoma cells led to the same conclusion. Indeed, even if the decrease in Km was statistically significant, it was small and could not account for the low Km transport component observed in dispersed β cells.

How can phosphorylation of the cytoplasmic tail affect transporter activity? Glucose transporters facilitate the diffusion of glucose molecules across biological membranes by a mechanism probably best described by the alternating conformation model. In this model the transporter has two mutually exclusive sugar binding sites, one present on the extracellular and the other on the intracellular surface. Binding of glucose to one site induces the transporter to switch to the opposite conformation, a process that is accompanied by movement of the transporters and that the transporters were present only at the cell surface. Fig. 7B shows the concentration-dependent increase in initial uptake rates by the SSST-A- and SSST-E-expressing insulinoma cells and the Eadie-Hofstee transformation of the data. Because both cell lines expressed identical amounts of transporters, the Vmax for both mutants can be directly compared from the Eadie-Hofstee plots. Table II presents the summary of the Km and Vmax values determined in four separate experiments. Altogether the data demonstrate that the mutations of the phosphorylation sites into glutamates induced a −40% decrease in the Vmax for 3-O-methyl glucose uptake compared with the alanine mutant. The difference in the Km values for both mutants, although significant when compared by t-test analysis, is rather low. These data compare well with the uptake measurements made with cell sorter purified β cells. Similar data have been obtained by comparing another set of GLUT2 SSST-A and SSST-E mutants.

Discussion

Phosphorylation of GLUT2 in cell sorter purified β cells was shown to be a rapid event initiated either by direct activation of adenylyl cyclase with forskolin or by GLP-1 binding to its receptor. That phosphorylation of the transporter was protein kinase A-dependent was further demonstrated by the inhibition of GLP-1-induced phosphorylation by the specific protein kinase A inhibitor H-89. Several sites of phosphorylation were identified in the cytoplasmic carboxyl-terminal tail of GLUT2 using fusion proteins phosphorylated in vitro by the catalytic subunit of protein kinase A. That the same sites are also substrates for PKA in intact cells was further demonstrated by expressing the wild-type GLUT2 and the mutant lacking the...
substrate across the membrane (reviewed in Carruthers (1990)). The molecular basis for this conformational change is not yet elucidated (Gould and Holman, 1993). However, reports have suggested that the carboxyl-terminal cytoplasmic tail may be important in determining the glucose transport function. For instance, deletion of this region in the structurally related GLUT1 transporter isoform generated a transporter that was locked in an inward facing conformation and was thus unable to transport glucose (Oka et al., 1990). Furthermore, replacing the cytoplasmic tail of GLUT1, a low $K_m$ (1-3 mM) high affinity transporter, with that of GLUT2 generated a chimera with increased $V_{max}$ and $K_m$ (Katagiri et al., 1992). In the GLUT4 transporter isoform phosphorylation in the cytoplasmic tail at serine 488 has also been reported to affect the intrinsic activity of the transporter (Reusch, 1994), although this view has been challenged (Lawrence, 1994). The present identification of PKA phosphorylation sites at serines 489 and 501/503 and threonine 510 and their role in decreasing the catalytic activity of the transporter further support an important role for the cytoplasmic tail in the control of transporter function. We further show that this process can be acutely regulated by hormones.

What is the possible role of GLUT2 phosphorylation on $\beta$ cell secretory activity? In the normal physiological situation, the rate of glucose transport in $\beta$ cells is 50–100-fold in excess over the rate of phosphorylation. GLUT2 thus appears to play mainly a permissive role, allowing an unrestricted access of glucose to glucokinase. A $\sim 40\%$ decrease in $V_{max}$ should therefore not impair the functioning of the glucose sensor. In addition, because GLP-1 stimulates insulin secretion, it cannot be easily understood how a decreased intrinsic activity could participate in the stimulatory effect. The possibility that GLUT2 may have an effect on the stimulation of insulin secretion, beside its transport function, has been proposed by Newgard and collaborators (Hughes et al., 1992, 1993; Ferber et al., 1994). When they transfected insulinomas with GLUT2 but not GLUT1, the cells recovered a glucose-dependent secretory response, although the rate of glucose metabolism was identical in both transfectants. Furthermore, when the effect of forskolin was tested on the secretory activity of the transfected cells, the increment in insulin secretion was higher in the GLUT2-expressing cells. Also, Valera et al. (1994) observed that a $\sim 70$–80% reduction in GLUT2 expression in $\beta$ cells of transgenic mice expressing GLUT2 antisense RNA leads to a decrease insulin secretory response and to diabetes. This suggests that even in conditions in which transport activity should not be limiting, $\beta$ cell diabetic dysfunctions develop. These data therefore suggest that GLUT2 may serve a signaling role in $\beta$ cells distinct from its transport activity. What is this signaling activity? Although mostly speculative at the moment, one can suggest that phosphorylation of GLUT2 may promote interactions with other protein components involved in the glucose transport function of insulinoma cells expressing either the SSST-E or SSST-A GLUT2 mutants.

**A**. quantitation and surface expression of both mutants in transfected insulinomas. Left, total cellular expression of SSST-E and SSST-A mutants in the cell lines used for uptake measurements as determined by Western blot analysis on increasing concentrations of cell lysates (in μg) as indicated. Duplicate determinations are shown. Right, the surface expression of the mutated transporters was confirmed by trypsin treatment of the cells at 4 °C, which converts GLUT2 into a faster migrating form. B. uptake was measured as described under “Material and Methods.” In the left panel is the rate of uptake by SSST-E (closed squares) and SSST-A (open circles) as a function of increasing substrate concentrations. Each point is the mean of three measurements. In the right panel is the Eadie-Hofstee transformation of the uptake data. Calculated $V_{max}$ and $K_m$ are 493 nmol/min/mg protein and 26 mM, respectively, for SSST-A and 305 nmol/min/mg protein and 22.5 mM, respectively, for SSST-E (see also Table II).

**FIG. 7.** 3-O-Methyl-glucose uptake by insulinoma cells expressing either the SSST-E or SSST-A GLUT2 mutants. In each experiment, the uptake kinetics were determined in parallel for both mutant forms of GLUT2. In parentheses is the $V_{max}$ for the glutamate form in the percentage of the alanine form. Significant differences between kinetic parameters for SSST-E and SSST-A mutants were determined by paired $t$ test analysis and were: $V_{max}$ $p = 0.023$; $K_m$, $p = 0.00967$. The average $V_{max}$ of SSST-E mutant is 61.5 ± 5.7% of the SSST-A $V_{max}$. $K_m$ and $V_{max}$ for untransfected RINm5F cells were 5.05 ± 0.77 mM and 17 ± 6.3 (nmol/min/mg protein), respectively.

| Experiment | Cells | $K_m$ (mM) | $V_{max}$ (nmol/min/mg) |
|------------|-------|------------|-------------------------|
| I          | SSST-E | 21         | 205 (62%)               |
|            | SSST-A | 35         | 329                     |
| II         | SSST-E | 12.8       | 142 (54%)               |
|            | SSST-A | 17.4       | 262                     |
| III        | SSST-E | 19.8       | 270 (68%)               |
|            | SSST-A | 25         | 393                     |
| IV         | SSST-E | 22.5       | 305 (62%)               |
|            | SSST-A | 26.2       | 493                     |

**TABLE II**

Kinetic parameters for 3-O-methyl glucose uptake by mutated GLUT2 in transfected RINm5F cells

Left panel, quantitation and 3-O-methyl-glucose uptake by insulinomacellsexpressingeithertheSSST-EorSSST-AGLUT2mutants. In the normal physiological situation, the rate of glucose transport in $\beta$ cells is 50–100-fold in excess over the rate of phosphorylation. GLUT2 thus appears to play mainly a permissive role, allowing an unrestricted access of glucose to glucokinase. A $\sim 40\%$ decrease in $V_{max}$ should therefore not impair the functioning of the glucose sensor. In addition, because GLP-1 stimulates insulin secretion, it cannot be easily understood how a decreased intrinsic activity could participate in the stimulatory effect. The possibility that GLUT2 may have an effect on the stimulation of insulin secretion, beside its transport function, has been proposed by Newgard and collaborators (Hughes et al., 1992, 1993; Ferber et al., 1994). When they transfected insulinomas with GLUT2 but not GLUT1, the cells recovered a glucose-dependent secretory response, although the rate of glucose metabolism was identical in both transfectants. Furthermore, when the effect of forskolin was tested on the secretory activity of the transfected cells, the increment in insulin secretion was higher in the GLUT2-expressing cells. Also, Valera et al. (1994) observed that a $\sim 70$–80% reduction in GLUT2 expression in $\beta$ cells of transgenic mice expressing GLUT2 antisense RNA leads to a decrease insulin secretory response and to diabetes.
signaling pathway. Phosphorylation of membrane receptors or associated proteins has been shown to be essential for several hormone or growth factor signaling pathways. The characterization of the phosphorylation sites and the generation of fusion proteins containing the variously mutated form of the transporter cytoplasmic tail may help identify proteins normally interacting with GLUT2. This could for instance be performed by testing the specific absorption of biosynthetically labeled islet proteins with the fusion proteins (Cosson and Letourneau, 1994) by screening of CDNA expression libraries with the fusion proteins (Skolnis et al., 1991) or by the yeast double hybrid system using the glutamate or alanine forms of the transporter cytoplasmic tail (Fields and Song, 1995; Chien et al., 1991).

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