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Substitution of conserved tyrosine residues in helix 4 (Y143) and 7 (Y293) affects the activity, but not IAPS-forskolin binding, of the glucose transporter GLUT4

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Abstract

Six tyrosine residues (Y28, Y143, Y292, Y293, Y308, Y432[1]) which are conserved in all mammalian glucose transporters were substituted for phenylalanine by site-directed mutagenesis, and mutant glucose transporters were transiently expressed in COS-7 cells. Glucose transport activity as assessed by reconstitution of the solubilized transporters into lecithin liposomes was reduced by 70% in the mutant Y143F and appeared to be abolished in Y293F, but was not affected by substitution of Y28, Y292, Y308 and Y432. In contrast, covalent binding of the photolabel ^{125}IAPS-forskolin was normal in all mutants. Stable expression of the mutants Y143F, Y293F, and Y292F in LTK cells yielded identical results. These data indicate that only two of the 6 conserved helical tyrosine residues, located in helices 4 and 7, are essential for full activity, but not for IAPS-forskolin binding of the GLUT4.

Key words: Glucose transport; IAPS-Forskolin; Insulin-regulated glucose transporter GLUT4

1. Introduction

Glucose transport into mammalian cells is catalyzed by a family of homologous proteins (GLUT1–GLUT4) exhibiting a striking tissue specificity [1]. All glucose transporter isoforms consist of 12 hydrophobic helices representing membrane spanning domains, intracellular C- and N-termini, and large extracellular and intracellular domains [2]. These proteins differ in some functional characteristics, e.g. K_m-values [3] and binding affinities of the inhibitory ligands cytochalasin B and forskolin [4]. The structural basis of the function of glucose transporters, and of their functional differences is not fully known, although a hypothetical model involving a conformational change within pore-forming helices is generally accepted [5].

Several amino acid residues in the GLUT1 have previously been exchanged by mutagenesis in order to define residues that are essential for transporter function. So far, these data have generated a complex picture of heterogenous requirements for transporter function and binding of ligands such as the bis-mannose derivative ATB-BMPA, cytochalasin B, and forskolin. Two tryptophane residues have been identified as potential contact sites of glucose and/or inhibitory ligands, e.g. cytochalasin B and forskolin: exchange of W388 in helix 10 markedly affected both glucose transport activity and affinity for inhibitory ligands, whereas the exchange of W412 in helix 11 exclusively reduced transporter activity [6,7]. Substitution of Q282 in helix 7 led to the suggestion that this domain is involved in the exofacial binding of sugars [8]. Furthermore, exchange of C421 decreased the inhibitory potency of cytochalasin B but failed to affect the transport activity of the GLUT1 [9].

All mammalian glucose transporters harbor 6 conserved tyrosines in their membrane spanning helices. Based on the hypothesis that the hydroxyl groups of the helical tyrosines might participate in the recognition of the hexose or in the interaction of the pore-forming helices, we have exchanged these tyrosine residues for phenylalanine. We have chosen the GLUT4 for this purpose, since this isoform exhibits an extraordinarily high affinity for the ligand forskolin [4]. Furthermore, transfected GLUT4 can easily be detected and quantitated in the presence of the constitutive GLUT1. The mutants were expressed in COS-7 cells, and their activity and photolabeling by IAPS-forskolin was assessed in an in vitro system of isolated membranes.

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Abbreviations: ATB-BMPA, 2-*N*4-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis(D-mannosyloxy)-2-propylamine; [^{125}I]IAPS-forskolin, 3-[^{125}I]-Iodo-4-azidophenetyl-amido-7-*O*-succinyldeacetyl-forskolin; GLUT1, erythrocyte/brain-type glucose transporter; GLUT4, adipocyte/muscle-type glucose transporter; PMSF, phenylmethylsulfonylfluoride; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

[1] In order to facilitate comparisons, the tyrosines in the GLUT4 are numbered corresponding with those in the GLUT1. The positions according to the numbering of the GLUT4 are (in parentheses): Y28 (40), Y143 (159), Y292 (308), Y293 (309), Y308 (324), Y432 (448).

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2. Materials and methods

2.1. Construction of mutant DNA and expression vectors

Mutants were generated by oligonucleotide-directed mutagenesis as described [10]. Single stranded DNA of rat GLUT4 [11] subcloned into pBluescript was used as the template. Mutated constructs were identified by sequencing and subcloned as *XbaI–KpnI* fragments from pBluescript into the mammalian expression vector pCMV which harbors a SV40 origin, a cytomegalovirus promoter, and a polyadenylation site [12].

2.2. Transfection of COS-7 cells

Transfection of COS-7 cells with glucose transporter cDNA was performed by a modification of the previously described procedure [12]. COS-7 cells were obtained from American Type Culture Collection (Rockville, MD) and were grown in Dulbecco's modified Eagle's medium (pH 7.4) containing 10% fetal calf serum, 20 mM HEPES, and 2 mM glutamine. The cells were harvested by trypsination 24 h before the transfection and were seeded onto 153 cm^2 culture dishes at a final density of 6.25×10^5 cells/dish. Four hours before the transfection, 25 ml fresh culture medium was added to the monolayer. DNA (25 μg/plate) was co-precipitated with 125 mM $CaCl_2$ in HBS-buffer (140 mM NaCl, 0.75 mM $Na_2HPO_4 \cdot 2H_2O$, 25 mM HEPES, pH 7.1) for 15 min in a final volume of 2.5 ml. The calcium phosphate–DNA suspension was added to the monolayers, and cells were incubated for 16 h at 37°C. Thereafter, the medium was exchanged for 7.5 ml 15% glycerol in HBS-buffer, and the cells were incubated for 3 min. The buffer was removed by aspiration, and monolayers were washed twice with prewarmed PBS (138 mM NaCl, 2.6 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). The cells were then incubated with fresh culture medium in an atmosphere of 5% CO_2 at 37°C for 48 h.

2.3. Transfection of LTK-cells

LTK cells were cultured in Eagle's minimum essential medium, pH 7.4, supplemented with 10% fetal calf serum, 2 mM glutamine, and 20 mM HEPES at 37°C in an atmosphere of 5% CO_2. Twenty-four hours before transfection, the cells were seeded in 83 cm^2 culture flasks at a density of 10^5 cells/flask and cultured overnight. After a 4 h incubation with fresh medium, 10 μg of plasmid DNA (pCMV, pCMV-GLUT4, pCMV-GLUT4-Y143F, pCMV-GLUT4-Y292F, pCMV-GLUT4-Y293F) and 1 μg of the plasmid harboring a geneticin resistance gene (pSV40-neo) were added. The plasmid DNA's were prepared by co-precipitation with 125 mM $CaCl_2$ in HBS-buffer (140 mM NaCl, 25 mM HEPES, 0.75 mM Na_2HPO_4, pH 7.1). After 16 h the transfection medium was removed, and each monolayer was treated for 2.5 min with 3 ml of 15% glycerol in PBS at room temperature. Cells were washed twice with HBS and cultured in fresh culture medium for 24 h. Stable transfectants were selected by culture in medium supplemented with 0.8 mg/ml G418 (Gibco BRL, Eggenstein, Germany) for 12 days.

2.4. Preparation of membrane fractions from transfected cells

The transfected cells were homogenized and fractionated as previously described [12] with a modification of a protocol previously employed in 3T3-L1 cells [13].

2.5. Immunoblotting of the GLUT4

Immunochemical detection and quantitation of the GLUT4 with antiserum against a dodekapeptide corresponding with the C-terminus of GLUT4 was performed as described previously [12].

2.6. Reconstitution of glucose transport activity from membrane fractions

Glucose transport activity reconstituted into lecithin liposomes was assayed as described previously [14] with minor modifications [15].

2.7. Photolabeling of glucose transporters with ^{125}I-IAPS-forskolin

IAPS-forskolin (3-[^{125}I]Iodo-4-azidophenetylamido-7-*O*-succinyldeacetyl-forskolin) was prepared as described [16] and was stored in ethanol solution at −20°C. Photolabeling was performed by a modification of a previously published procedure [17]. Samples of plasma membranes (100 μl) containing 30 μg of membrane protein were incubated with [^{125}I]IAPS-forskolin (2 μl, 0.4 μCi, final concentration 50 nM) for 30 min on ice. The samples were photolyzed by 6 flashes (setting on 1,000 W·s) with the high pressure mercury lamp of the LIZZY photolysis flash (Raytest, Straubenhardt, Germany). The reaction was immediately quenched by addition of 10 μl 10% β-mercaptoethanol, and the samples were diluted with 1 ml of ice-cold Tris-buffer (20 mM). Membranes were separated by centrifugation in a refrigerated microfuge (13,500 rpm, 30 min), and the resulting pellets were separated by SDS-PAGE (10% gels). The gels were dried and autoradiographed for 2–7 days.

3. Results

Since previous experiments had indicated that glucose transport activity in intact COS-7 cells does not increase proportionally with the expression of a transiently transfected GLUT4 or GLUT1 [12], we have chosen to assay the glucose transport activity of the mutants in an in vitro reconstitution assay. Membranes from transfected cells were solubilized, mixed with lecithin liposomes, and subjected to the freeze-thaw sonication procedure [14,15]. Control experiments were performed in which different amounts of DNA were employed in order to determine the optimum conditions of the transfection, and to assess the validity of the transport assay (data not shown). The expression of the wild-type GLUT4 increased gradually when amounts of DNA between 1 and 7 μg per culture dish (57 cm^2) were employed in the

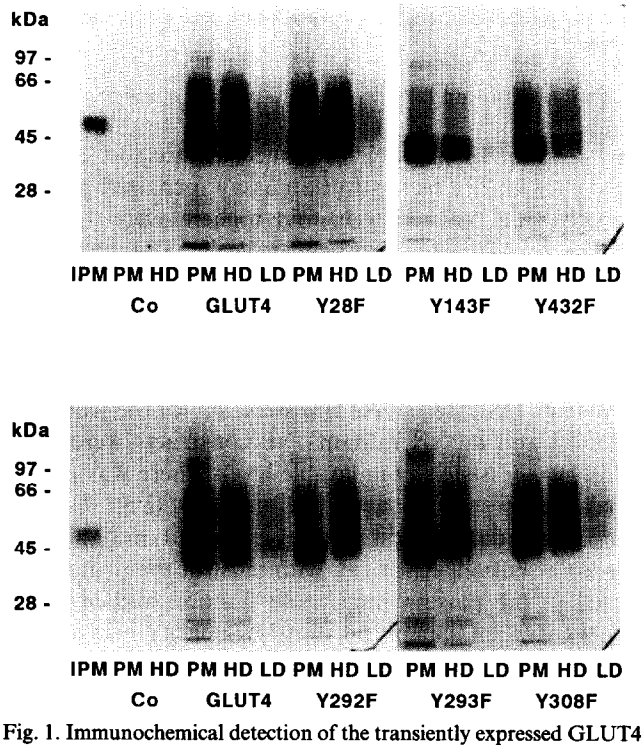

Fig. 1. Immunochemical detection of the transiently expressed GLUT4 mutants in membrane fractions from COS-7 cells. COS-7 cells were transfected with the GLUT4 mutant DNA subcloned into pCMV (25 μg/plate). After 48 h, cells were homogenized and fractionated by differential fractionation as described. Samples of membrane proteins were separated by SDS-PAGE and immunoblotted. PM, plasma membranes; HD, high-density microsomes; LD, low-density microsomes. For comparison, samples of plasma membranes from insulin-treated adipocytes (IPM) were run in parallel.

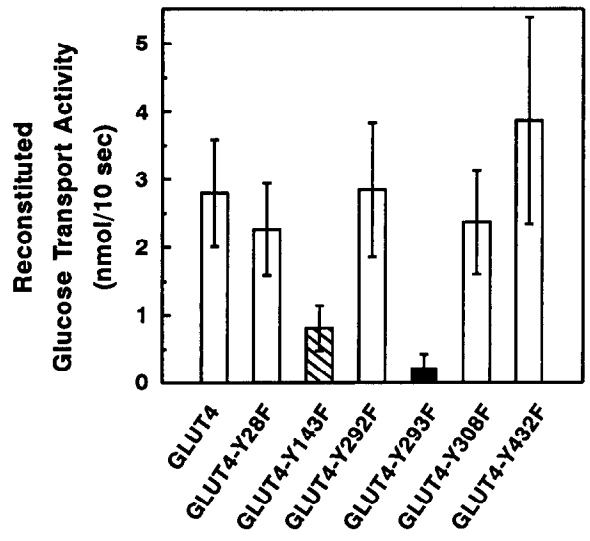

Fig. 2. Reconstituted glucose transport activities of GLUT4 mutants transiently expressed in COS-7 cells. Samples (60 μg) of plasma membranes from COS-7 cells transfected with the indicated mutant DNA (25 μg/plate) were solubilized, and glucose transport activity was reconstituted and assayed as described in section 2. Glucose uptake rates in controls (transfection with bland vector) were routinely 15–25% of those in membranes containing wild-type GLUT4. After subtraction of the control rates, the data were corrected for different amounts of GLUT4 or GLUT4 mutants present in the membranes as assayed by immunoblotting in parallel samples. The data represent means ± S.E. of three independent transfection experiments, each assayed in quadruplicate.

transfection. In parallel, the glucose transport activity assayed with the reconstitution assay increased proportionally, and reached 6–10-fold higher levels than the activity reconstituted from control cells. The amount of DNA giving rise to maximum GLUT4 expression (10 μg/2.5 × 10^5 cells) was used routinely in all experiments and adjusted to the larger culture dishes employed subsequently (25 μg/plate with 6.25 × 10^5 cells).

When COS-7 cells were transfected with the wild type GLUT4 and the six mutants, all constructs were transiently expressed in comparable levels; there was no apparent difference between the mutants (Fig. 1). The subcellular distribution of the expressed mutants appeared essentially identical, with most of the protein detected in the plasma membrane fraction and approximately 10% of that found in a low-density microsomal fraction. Fig. 2 illustrates a comparison of the reconstituted glucose transport activities of six GLUT4 mutants. In these experiments, transfection with wild-type GLUT4 routinely produced a 4–5-fold increase of reconstituted glucose transport activity over basal values. Equal amounts of membrane protein were used in the assays, and basal rates (membranes from transfection with bland pCMV vector) were subtracted from the results. Thereafter, the data were corrected for slight differences in the levels (immunoreactivity) of the expressed glucose transporters. The results of three independent transfections and membrane preparations indicate that the mutants Y28F, Y292F, Y308F and Y432F induced an increase in glucose transport activity comparable to that of the wild type. In contrast, the glucose transport activity of Y143F was markedly reduced (30% of the wild-type GLUT4), whereas Y293F completely failed to increase the reconstituted transport activity (Fig. 2).

In order to confirm these results in an independent expression system, the wild-type GLUT4 and three of the mutants (Y143F, Y292F, Y293F) were transfected into LTK fibroblasts, and clones stably expressing the constructs were isolated. The reconstituted glucose transport activity was 7–10-fold higher in cells expressing the wild-type GLUT4 than in control clones transfected with bland vector. Values from control cells were subtracted, and the increments were corrected for differences in GLUT4 immunoreactivity assayed in separate samples. Table 1 summarizes the data obtained with the different mutants after this correction: the wild-type GLUT4 and the mutant Y292F produced an essentially identical increase of the reconstituted glucose transport activity. In contrast, the activity of Y143F was only 25% of that of the wild type, whereas Y293F appeared completely inactive. These data fully agree with the results obtained with the COS-7 cells.

Several hydrophobic agents bind to glucose transporters and inhibit their activity. Among these, the photoreactive derivative IAPS-forskolin exhibits the highest affinity (IC_{50} of inhibition of glucose transport 3 × 10^{-8} [16]). In order to test whether binding of the photolabel was affected in any of the mutant glucose transporters, membranes from transfected COS-7 cells were photolyzed in the presence of tracer concentrations (0.4 μCi/sample) of ^{125}I-IAPS-forskolin. As is illustrated in Fig. 3 (upper panel), covalent labeling of the GLUT4 resulted in a broad signal (45–50 kDa) reflecting the hetero-

Table 1
Reconstituted glucose transport activity of GLUT4 mutants stably expressed in LTK cells

	Reconstituted transport activity (nmol/10 min)
wild type GLUT4	0.95 ± 0.13
Y143	0.22 ± 0.04
Y292F	1.01 ± 0.12
Y293F	not detectable

Samples (60 μg) of plasma membranes from LTK cell clones stably expressing DNA were solubilized, and glucose transport activity was reconstituted and assayed as described in section 2. Glucose uptake rates in controls (transfection with bland vector) were routinely 15–25% of those in membranes containing wild-type GLUT4. After subtraction of the control rates, the data were corrected for different amounts of GLUT4 or GLUT4 mutants present in the membranes as assayed by immunoblotting in parallel samples. The data represent means ± S.E. of quadruplicate samples from a representative experiment.

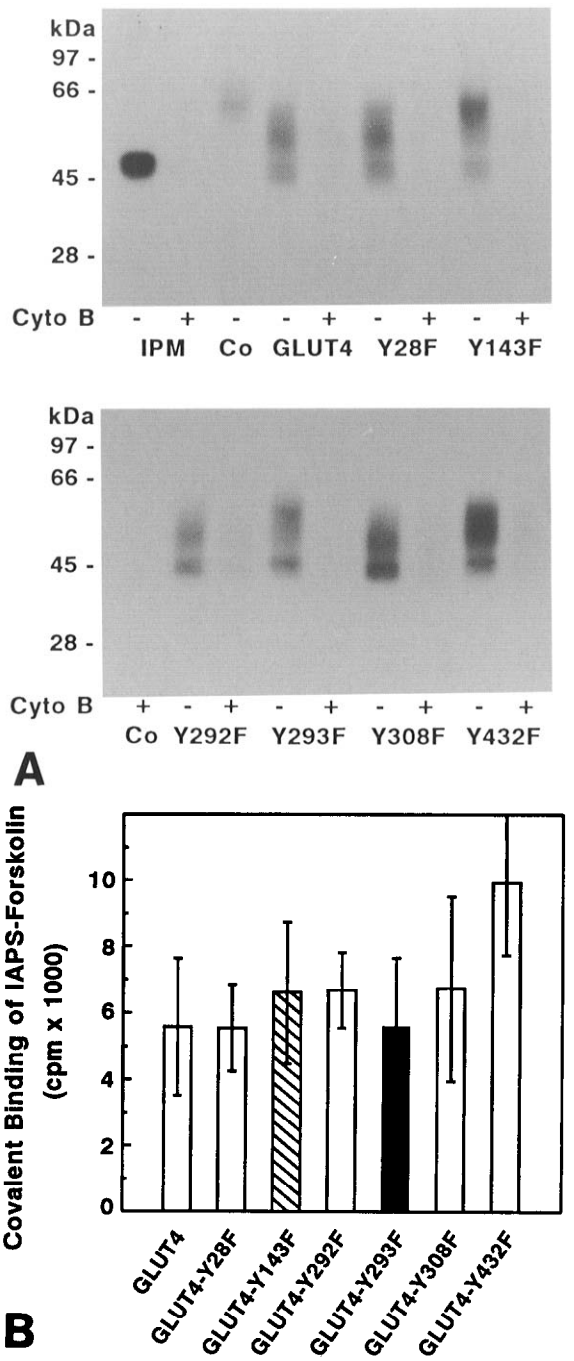

Fig. 3. Photolabeling of GLUT4-mutants transiently expressed in COS-7 cells with ^{125}I-IAPS-forskolin. Samples (30 μg) of plasma membranes from COS-7 cells transfected with the indicated mutant DNA (25 μg/plate) were photolyzed in the presence of ^{125}IAPS-forskolin as described in section 2. The membranes were isolated by centrifugation and separated by SDS-PAGE. Gels were dried and autoradiographed for 1–4 days. Cyto B, presence of 25 μM cytochalasin B. For comparison, samples of plasma membranes from insulin-treated adipocytes (IPM) were run in parallel. Upper panel: autoradiograph of a representative experiment. Note that each sample contained an identical amount of total protein with a slightly different amount of transporter protein. Lower panel: the radioactivity incorporated into the 45–55 kDa bands were determined by cutting and scintillation counting, and was normalized for the different amounts of GLUT4 or GLUT4 mutants as assayed by immunoblotting in parallel samples. The data represent means ± S.E. of 3 independent transfection experiments.

genous glycosylation of the transporter. The labeling was inhibited by glucose (not shown) or cytochalasin B. As can be judged from a comparison with a control (transfection with bland pCMV), labeling of the endogenous GLUT1 results in a much weaker signal which is distinguishable from the transfected GLUT4 because of its lower electrophoretic mobility (apparent molecular weight 60 kDa). Thus, the results were quantitated by cutting and counting of the labeled band, and were normalized per GLUT4 immunoreactivity assayed in parallel samples (Fig. 3, lower panel). This normalization indicated that the labeling of all mutants was comparable with that of the wild-type GLUT4. In contrast to the results obtained in the reconstitution assay, photolabeling of Y143F and Y293F was indistinguishable from that of the wild type. Furthermore, photolabeling of the mutants Y143F, Y292F and Y293F stably expressed in LTK cells was normal (data not shown).

4. Discussion

The present data indicate that out of the 6 conserved helical tyrosine residues only two, Y143 and Y293, are required for the transport activity of the GLUT4. Y293 is conserved in all known mammalian hexose transporters, yeast glucose transporters, plant hexose–proton symporters [18], and some bacterial sugar–proton symporters. Y143 is conserved in all mammalian and some yeast, plant, and bacterial hexose transporters. Furthermore, Y143 is located within a highly conserved domain in helix 4 (VPMYV/IGEV/I) of the mammalian glucose transporters. It appears reasonable to conclude that the hydroxyl groups of these two tyrosines provide hydrogen bonds for the formation of the pore-forming complex of helices or represent contact sites for the substrate. The present data do not allow a distinction between these possibilities. However, the mutations have not produced a gross alteration of the quarternary structure of the protein, since binding of the label IAPS-forskolin to the mutant transporter was normal. Furthermore, when our manuscript was in preparation, a study was published showing that substitution of Y293 in the GLUT1 abolished the binding of the mannose-derived photolabel ATB-BMPA [19]. Thus, it appears safe to conclude that the loss of activity produced by mutation of Y293 reflects the specific alteration of an amino acid involved in transporter function, rather than a non-specific effect on the conformation of the protein.

The current hypothesis on glucose transporter function comprises a transport pathway formed by membrane spanning helices [5]. Specific permeation of glucose takes place by exofacial binding of the hexose, an induced conformational change of the pore-forming region, and transfer of the hexose to an endofacial contact site. It has been suggested that a domain in helix 7 repre-

sents the exofacial recognition site of the hexose, since exchange of Q282 abolished the binding of the impermeant, i.e. exofacial, photolabel ATP-BMPA [8]. Y293 is located at the exofacial end of helix 7 and might be involved in the exofacial recognition of glucose, or be required for the conformational change of the transporter protein. Interestingly, a computerized assessment of the location of amino acids in helix 7 (HELWHEEL program) indicates that the residues Q282 and Y293 face the same side of the helix.

The plant product forskolin is considered a specific ligand of mammalian glucose transporters (K_d approximately 10^{-7} M in GLUT4, 10^{-6} M in GLUT1, no specific binding detectable in GLUT2 [4]). Its binding to glucose transporters is inhibitable by glucose or cytochalasin B [20], suggesting that cytochalasin B, glucose and forskolin have common binding sites in the transporter protein. The present data indicate that the structural requirements for glucose transport activity and forskolin binding, and probably also the respective contact sites, can partially be dissociated: whereas Y143 and Y293 appear to be necessary for the function of the GLUT4, exchange of these residues failed to affect the binding of the forskolin-derived photolabel. Thus, the structural requirements determining glucose transport activity and IAPS-forskolin binding overlap only partially, e.g. for W388 [6,7], but not completely. This finding might indicate that forskolin, like cytochalasin B [5], binds exclusively to the internal contact site of glucose.

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