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Glucose transport activity and ligand binding (cytochalasin B, IAPS-forskolin) of chimeric constructs of GLUT2 and GLUT4 expressed in COS-7-cells

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Abstract

Chimeric constructs of glucose transporters GLUT2 and GLUT4 were transiently expressed in COS-7 cells in order to determine regions of the proteins responsible for their differences in activity and ligand binding. Exchange of the C-terminal tail (aa 479-509) of GLUT4 failed to affect glucose transport activity assayed at 1 mM glucose or ligand binding (cytochalasin B, IAPS-forskolin). In contrast, exchange of the C-terminal half of GLUT4 (aa 222-509) for that of GLUT2 markedly reduced ligand binding (K_d of cytochalasin B binding 1.88 ± 0.2 μM vs. 0.21 ± 0.06 in the wild-type GLUT4), and moderately (25%) reduced glucose transport activity. These data support the conclusion that the domains determining differences in ligand binding between GLUT4 and GLUT2 are located in the C-terminal half of the glucose transporters.

Keywords: Glucose transport; 3-[^{125}I]Iodo-4-azidophenetylamido-7-O-succinyldeacetyl-forskolin; Insulin-regulated glucose transporter GLUT4; Cytochalasin B

1. Introduction

The carrier proteins mediating facilitated diffusion of glucose (GLUT1-4) are inhibited by several structurally diverse agents, e.g., cytochalasin B [1], forskolin [2], or methylxanthines [3]. These inhibitory ligands appear to share binding sites on the glucose transporters with glucose, because their binding is inhibited by glucose in a competitive manner [1,4–6]. The binding characteristics of inhibitory ligands have been used by several investigators to identify domains of the glucose transporter which are involved in the recognition and translocation of the hexose. Employing photoreactive glucose derivatives and radiolabeled cytochalasin B, Holman and Rees have located the site of covalent labeling with glucose to helix 9, and that of cytochalasin B to the internal site of helix 10 [7]. Similarly, the site of covalent labeling with the photoreactive forskolin derivative IAPS-forskolin was determined by labeling, partial proteolysis and fragment identification with specific antisera. In these experiments, the site of covalent labeling of the GLUT4 was located between helices 7 and 9 [8]. Other researchers have found labeling of helix 10, but have confirmed that only the C-terminal half, and none of the first six helices, is labeled [9].

The glucose transporter isoforms differ markedly in their affinity, i.e. their K_m for hexoses [10–13], but also in the K_d of the inhibitory ligands [6,13]. Of the known isoforms, GLUT4 exhibits the lowest K_m for 3-O-methylglucose under equilibrium exchange conditions [10] and the highest affinity for both cytochalasin B and forskolin

Abbreviations: [^{125}I]IAPS-forskolin, 3-[^{125}I]Iodo-4-azidophenetylamido-7-O-succinyldeacetyl-forskolin; GLUT1, erythrocyte/brain-type glucose transporter; GLUT2, liver-type glucose transporter; GLUT4, adipocyte/muscle-type glucose transporter; PMSF, phenylmethylsulfonylfluoride; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

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[6]. GLUT2 exhibits the highest K_m for glucose and 3-O-methylglucose [10,12]; it binds cytochalasin B with low affinity [14], and fails to bind forskolin [6]. These parallel differences in the affinities for glucose and forskolin appear to reflect structural differences that determine important domains in the carrier proteins. Recently, chimeric constructs of human GLUT2/GLUT4 have been expressed in *Xenopus* oocytes in order to identify the regions in these transporters which are responsible for their different affinities to glucose [15]. Since this expression system does not provide sufficient amounts of transporter protein for assays of ligand binding, we have expressed the chimeric constructs in COS-7 cells, and have assessed their transport activity as well as the binding of cytochalasin B and IAPS-forskolin. The data indicate that the domains determining the affinities of ligand binding are located in the C-terminal half of the carrier protein, and do therefore support the previous conclusion that both ligands bind predominantly, if not exclusively, to this part of the glucose transporter [7,8]. Furthermore, the data argue against a role of the C-terminal tail in activity and ligand binding of the transporters.

2. Materials and methods

2.1. Construction of chimeric glucose transporter cDNA and expression vectors

Constructs were generated as described [15] by a PCR-based cloning approach, sequenced and subcloned as *Xba*I-*Sma*I fragments from pBluescript (GLUT4, GLUT4(1-82)/GLUT2(98-524), GLUT2(1-98)/GLUT4(82-509), GLUT4(1-222)/GLUT2(238-524)), as a *Sma*I-*Hin*dIII fragment from pGOV (GLUT4(1-479)/GLUT2(497-524)), or as a *Bgl*II fragment (GLUT2) from pSP64T [13] into the mammalian expression vector pCMV which harbors a SV40 origin, a cytomegalovirus promoter, and a polyadenylation site [16].

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 [16]. COS-7 cells were obtained from American Type Culture Collection (Rockville, USA, cat. no.: CRL 1651) 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, 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 pre-warmed 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. Preparation of membrane fractions from transfected cells

The transfected cells were homogenized and fractionated as previously described [16] with a modification of a protocol previously employed in 3T3-L1 cells [17].

2.4. Immunochemical detection of the glucose transporters

Immunochemical detection and quantitation of the constructs was performed with antisera against dodekapeptides corresponding with the C-terminus of GLUT4 or the GLUT2, and the N-terminus of the GLUT4. Antisera against the GLUT4 were characterized by cross- immunoprecipitation and blotting, antisera against the GLUT2 were characterized with human liver membranes. All electrophoresis and blotting procedures were performed as described previously [16].

2.5. Reconstitution of glucose transport activity from membrane fractions

Glucose transport activity reconstituted into lecithin liposomes was assayed as described previously [18] with minor modifications [19].

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

IAPS-forskolin (3-[^{125}I]iodo-4-azidophenetylamido-7-O-succinyl-deacetyl-forskolin) was prepared as described [9] and was stored in ethanol solution at −20°C. Photolabeling was performed by a modification of a previously published procedure [8]. Samples of plasma membranes (30 μg membrane protein) in 100 μl of Tris-buffer (20 mM, pH 7.4) 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 1000 Watt * 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.

2.7. Assay of equilibrium cytochalasin B binding

Cytochalasin B binding was determined by a modification of the previously published method [20]. Samples of plasma membranes (20 μg protein) in 200 μl Tris-buffer (20 mM, pH 7.4) were incubated with 0.05 μCi [^{3}H]cytochalasin B (NEN, spec. act. 9.8 Ci/mmol), 0.04 μCi [^{14}C]urea (Amersham, 56 mCi/mmol), and the desired concentrations of unlabeled cytochalasin B (1, 10, 100, 1000 and 10000 nM) for 10 min on ice. Membranes were separated by centrifugation in a refrigerated microfuge (13500 rpm, 30 min). After removal of the supernatants, the tips of the tubes were cut off, and the pellets were solubilized in 0.5 ml tissue solubilizer (BTS-450, Beckman, Munich, Germany). Bound and free radioactivity were determined in a water-compatible scintillation cocktail (Ready Protein, Beckman). Values of the bound fraction were corrected for trapped buffer by subtraction of counts from [^{14}C]urea. K_d values were determined graphically as described previously [21,22].

3. Results

Four chimeric glucose transporters (Fig. 1) were constructed by a PCR-based approach which introduced suitable restriction sites into the regions to be fused. By this strategy, the C-terminal cytoplasmatic tail (GLUT4/2T), the C-terminal half (GLUT4/2L) or the first helix including intra- and extracellular loops (GLUT2/4) of the GLUT2 were introduced into the GLUT4. In addition, the first helix of GLUT4 was introduced into GLUT2 (GLUT4/2).

All constructs were subcloned into the mammalian expression vector pCMV and transiently expressed in COS-7 cells. As is illustrated in Fig. 2, immunoreactive transporter protein was detected in membranes from cells transfected with each of the chimeric constructs or with the wild-types. No immunoreactivity was detected in cells transfected with the bland vector. All transporters migrated as broad smears because of their heterogenous glycosylation [16,23]. However, there were differences in their electrophoretic mobilities: GLUT4, GLUT4/2, GLUT4/2L and GLUT4/2T produced a similar spectrum of bands between 45 and 66 kDa, whereas the GLUT2 migrated as a somewhat sharper signal at 55 kDa with two additional bands (29 and 34 kDa) probably generated by proteolysis. Similarly, the GLUT2/4 product appeared sensitive to proteolysis, since a major portion of it migrated with an apparent molecular weight of 24–28 kDa (Fig. 2). The subcellular distribution of the chimeric constructs and the wild-type appeared essentially identical with 50% of the transporters in the plasma membrane fraction, 40% in the high-density microsomal fraction, and approx. 10% of the total recovered transporter found in a low-density microsomal fraction.

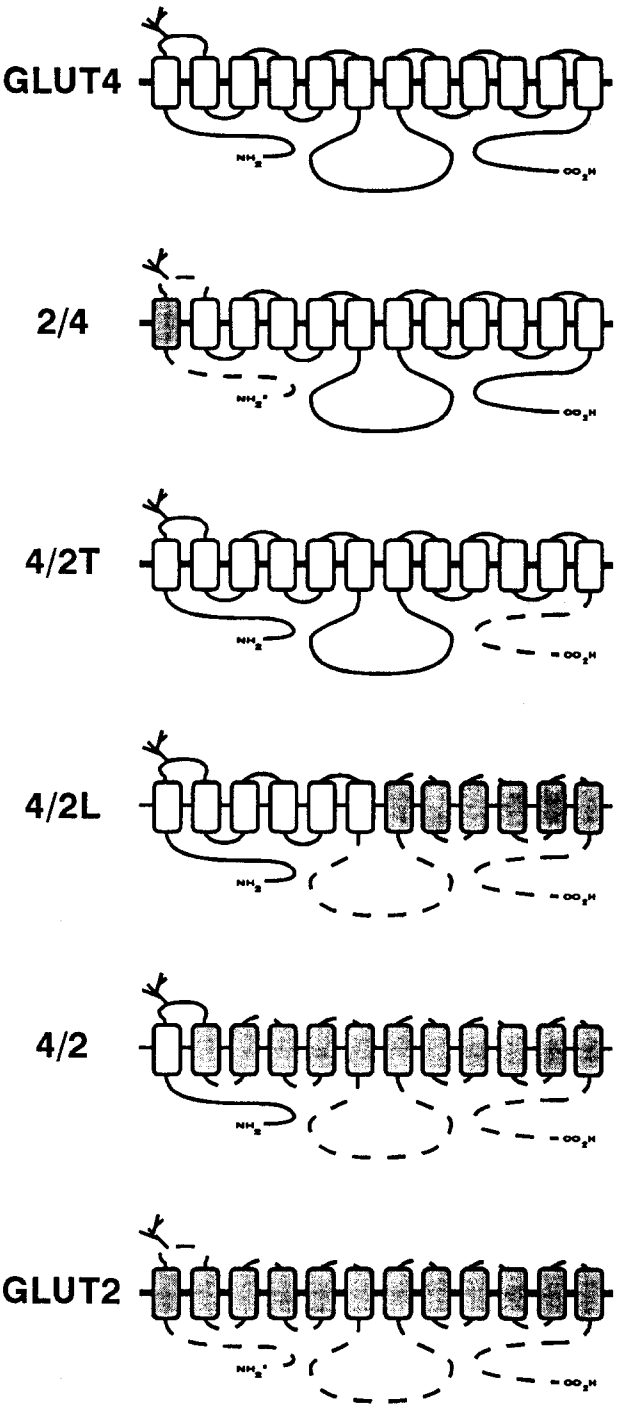

Fig. 1. Schematic presentation of chimeric glucose transporters tested in the present study. Helical domains of GLUT4 are represented by open symbols, helices of GLUT2 are shaded. Loop domains of GLUT2 are depicted by broken lines. Numbering of the predicted amino acids in the constructs: GLUT4, 1-509; GLUT2/4, GLUT2(1-98)/GLUT4(82-509); GLUT4/2T, GLUT4(1-479)/GLUT2(497-524); GLUT2/2L, GLUT4(1-222)/GLUT2(238-524); GLUT4/2, GLUT4(1-82)/GLUT2(98-524); GLUT2, 1-524.

Fig. 3 illustrates a comparison of the reconstituted glucose transport activities of wild-type and chimeric transporters. In order to correct for differences in the expression, a normalization procedure was employed which

took advantage of the epitope-specific antisera against GLUT4 (N-terminus and C-terminus) and GLUT2 (C-terminus). Immunoblots of the membranes with these antisera allowed a normalization of all constructs with either N- or C-terminus of the GLUT4; the expression of the wild-type GLUT2 was normalized by comparison with the constructs GLUT4/2T and GLUT4/2L. Transfection of COS-7 cells with GLUT4 routinely produced a 4- to 8-fold increase in the reconstituted glucose transport activity (Fig. 3). The effect of the construct GLUT4/2T, in which the C-terminus was exchanged, was indistinguishable from that of GLUT4. The exchange of the C-terminal half (in GLUT4/2L) produced a small (20–25%) but significant reduction ($P < 0.05$, paired t-test of 6 different transfections) of the glucose transport activity of the GLUT4. The wild-type GLUT2, in contrast, showed a much lower transport activity in the reconstitution assay than GLUT4; transfection of its cDNA produced only a 2.5-fold increase in uptake rates. As is illustrated in the lower panel of Fig. 3, transfection with the construct GLUT2/4 failed to increase the reconstituted glucose transport activity. Because of the partial degradation of the protein as observed in the immunoblots (see Fig. 2), this finding was not entirely unexpected. Surprisingly, the construct GLUT4/2 also appeared to generate an inactive protein; reconstituted

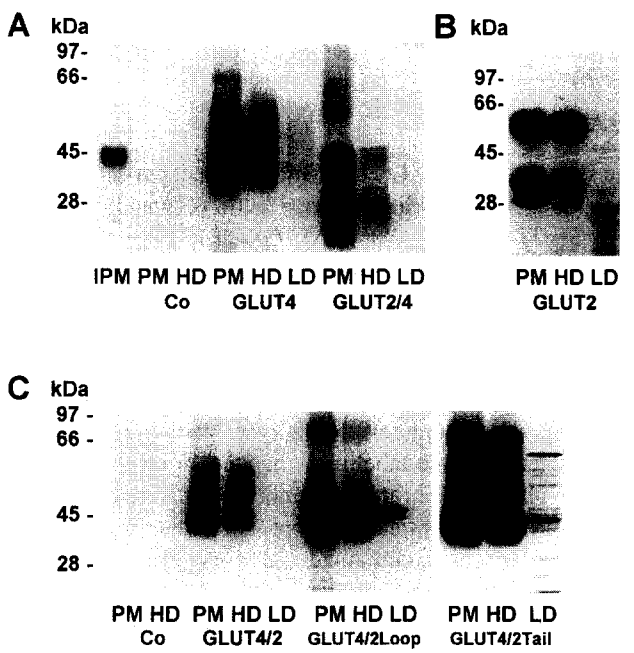

Fig. 2. Immunochemical detection of chimeric glucose transporters transiently expressed in COS-7 cells. COS-7 cells were transfected with the indicated constructs and fractionated as described. Samples of membrane proteins (2.5 μg/lane (A) or 10 μg/lane (B, C)) were separated by SDS-PAGE and immunoblotted. For comparison, membranes from adipocytes (IPM) were run in parallel. PM, plasma membranes; HD, high-density microsomes; LD, low-density microsomes. Co, transfection with bland vector. Immunochemical detection was performed with antiserum against the C-terminus of GLUT4 (panel A), GLUT2 (panel B), or the N-terminus of GLUT4 (panel C).

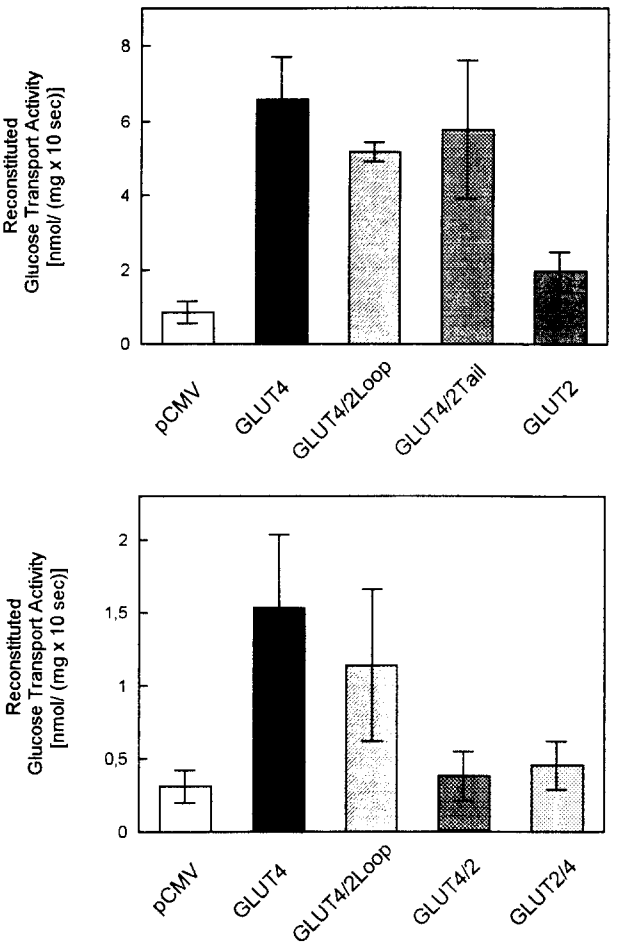

Fig. 3. Reconstituted glucose transport activity in membranes from COS-7 cells transfected with chimeric glucose transporter constructs. Samples of plasma membranes (60 μg) from COS-7 cells transfected with the indicated constructs were solubilized and reconstituted into lecithin liposomes as described. Glucose uptake rates were assayed, and the data were corrected for minor differences in the GLUT immunoreactivity as determined with antisera against the N-terminus of GLUT4 and the C-terminus of GLUT2 and GLUT4. Upper and lower panel represent data from two separate series as means ± S.D. of three independent transfections.

transport activity from cells transfected with this construct was not different from that of the controls. It should be noted that this construct increased glucose transport activity in *Xenopus* oocytes [15].

In order to assay equilibrium [^{3}H]cytochalasin B binding, large-scale transfections were performed with GLUT4, GLUT4/2T and GLUT4/2L. The other chimeric constructs were tested in preliminary assays (at tracer cytochalasin B concentration only), and no specific binding exceeding that of control samples was detected. The assays were run at 5 different cytochalasin B concentrations, and Scatchard plots (Fig. 4) were evaluated by subtraction of each data point of a control curve (transfection of bland vector) from the data of the constructs along radial axes. This subtraction generated linear plots (filled symbols)

which give both the K_d of cytochalasin B binding and the total number of sites. Fig. 4 illustrates a representative experiment indicating that the affinity of cytochalasin B binding was identical in the wild-type GLUT4 and the construct GLUT4/2T, whereas it was markedly reduced in the construct GLUT4/2L. Identical results were obtained in 3 independent additional transfections (means ± S.E. of K_d: GLUT4, 0.21 ± 0.06 μM; GLUT4/2T, 0.18 ± 0.04; GLUT4/2L, 1.88 ± 0.2; $n = 4$).

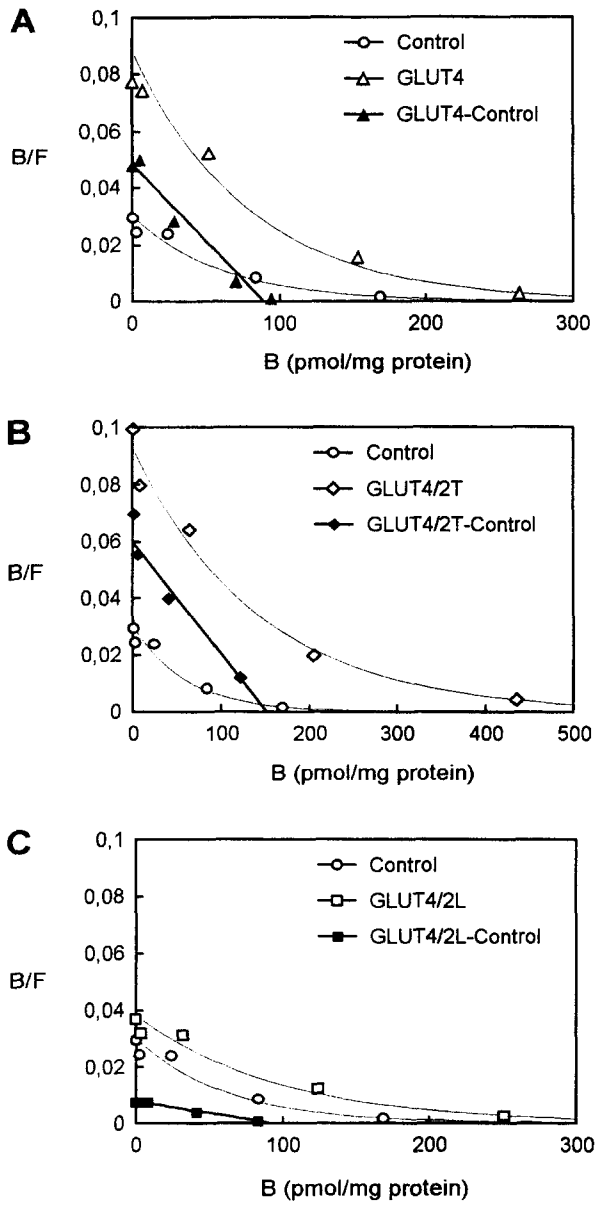

Fig. 4. Binding of cytochalasin B to chimeric glucose transporters expressed in COS-7 cells. Samples of plasma membranes (20 μg) from COS-7 cells transfected with the indicated constructs were incubated as described with [^{3}H]cytochalasin B and unlabeled ligand. Bound and free tracer fractions were separated by centrifugation, and the data were calculated and presented as Scatchard plots. In order to correct for non-specific binding and constitutive GLUT1, differences between transfections with constructs and bland vector were determined by subtraction along radial axes [21,22]; the resulting data points (filled symbols) were evaluated as linear Scatchard plots. A, GLUT4; B, GLUT4/2T; C, GLUT4/2L. Means ± S.E. of K_d (4 independent transfection experiments): GLUT4, 0.21 ± 0.06 μM; GLUT4/2T, 0.18 ± 0.04; GLUT4/2L, 1.88 ± 0.2). Means ± S.E. of number of binding sites: GLUT4, 0.11 ± 0.04 nM/mg of protein; GLUT4/2T, 0.094 ± 0.02; GLUT4/2L, 0.08 ± 0.003; $n = 4$).

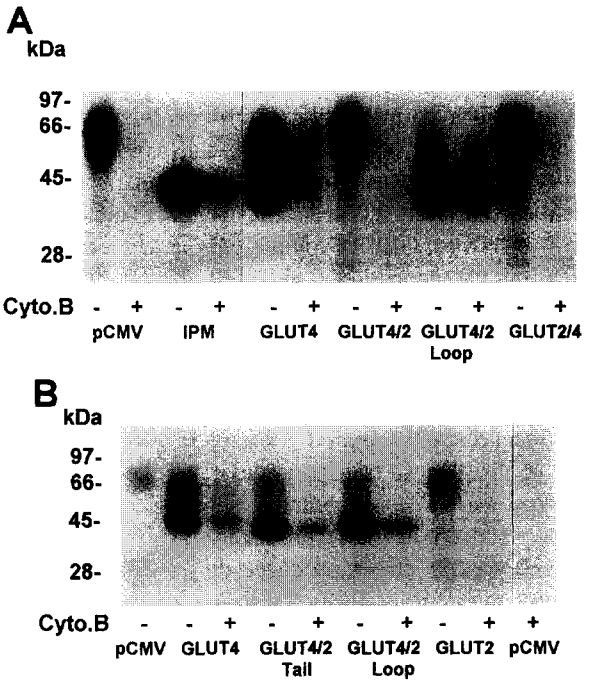

Fig. 5. Labeling of chimeric glucose transporters expressed in COS-7 cells with IAPS-forskolin. Cells were transfected with the indicated constructs, and samples of plasma membranes containing equal amounts of protein (30 μg) were photolysed with IAPS-forskolin in the presence or absence of cytochalasin B (25 μM). Note that the membrane preparations contained approx. 3-fold more GLUT4/2L than GLUT4 and GLUT4/2T. Data in the upper and lower panel were from two different series, each being representative for 3 independent transfections. pCMV, control transfections with bland vector; IPM, plasma membranes from adipocytes.

In order to assess the binding of IAPS-forskolin to the constructs expressed in COS-7 cells, membranes were photolysed with [^{125}I]IAPS-forskolin in the presence or absence of excess cytochalasin B (25 μM). In all experiments, equal amounts of protein were photolyzed, because the efficiency of the labeling is dependent on this parameter. The abundance of the immunoreactive transporters (not shown) was identical except in membranes with GLUT4/2L which contained only one third of the immunoreactivity detected in the samples with GLUT4. As is shown in Fig. 5, the photolabeled wild-type GLUT4 produced the anticipated broad signal between 45 and 66 kDa. A portion of this signal is generated by the constitutive GLUT1, as was demonstrated by transfection with bland vector. Transfection with the construct GLUT4/2L produced a similar incorporation of the photolabel. Thus, when normalized per amount of transporter, the photolabeling of GLUT4/2L appears lower than that of the wild-type GLUT4 because of its approx. 3-fold higher abundance in the photolabeled membranes. When cells

were transfected with GLUT4/2 and GLUT2/4, only the constitutive glucose transporter was labeled (signal at 66 kDa). In a second series of transfections and labeling reactions, the GLUT4/2T was studied and compared with wild-type GLUT4 and GLUT2. As is shown in the lower panel of the figure, there was no difference in the signals between GLUT4 and GLUT4/2T. In contrast, no specific signal was observed that corresponded with the GLUT2. In summary, the data indicate that GLUT4 and GLUT4/2T are labeled to a similar extent. In contrast, labeling is significantly reduced, but still detectable, with GLUT4/2L. Transfected GLUT2, however, was not labeled to any detectable extent.

4. Discussion

The present data indicate that binding of inhibitory ligands cytochalasin B and forskolin to the GLUT4 is markedly reduced, when the C-terminal half of the protein is exchanged for that of the GLUT2. Since this construct (GLUT4/2L) was still capable of catalyzing the transport of glucose, the reduction in ligand binding does not appear to reflect a non-specific conformational change that completely destroyed the functions of the protein. In contrast to the exchange of the whole C-terminal half of the protein, the exchange of the C-terminal tail (construct GLUT4/2T) was without any detectable effect on either transport activity or ligand binding. Therefore, the data localize the sites of high-affinity binding of cytochalasin B and forskolin into the C-terminal half of the transporter protein, but exclude the participation of its intracellular tail segment. This conclusion is in good agreement with previous evidence based on photolabeling and proteolytic cleavage of the labeled proteins [7,8]. Furthermore, two tryptophan residues in the C-terminal half of the GLUT1 (W388 and W412) have previously been identified as putative contact sites for glucose, cytochalasin B and forskolin [24–26]. Finally, when the two halves of the GLUT1 are expressed together as separate proteins in SF9 cells, only the C-terminal half is labeled with cytochalasin B and a photoreactive bismannose compound [27]. Taken together, these data strongly suggest that the binding sites of inhibitory ligands are predominantly, if not exclusively, located in the C-terminal half of the glucose transporter. It should be noted, however, that two residues in the N-terminal half (Y143 [23] and Q161 [28]), which appear necessary for the full transport activity and cytochalasin B binding, have recently been identified. Thus, it cannot entirely be excluded that there are additional contact sites in the N-terminal half which are not detected by a photolabeling approach.

The glucose transport activity of the construct GLUT4/2L as assayed in the present study was only moderately lower than that of the wild-type GLUT4. This finding was unexpected, since cytochalasin B binding was nearly abolished in this construct. We had anticipated parallel changes of glucose transport activity and cytochalasin B binding, because we assumed the ligands to bind to domains responsible for the affinity to glucose, i.e., the K_m. Moreover, it was recently shown that the K_m of the GLUT4/2L, as determined in *Xenopus* oocytes, indeed approached that of the GLUT2 [15], indicating that the C-terminal half of the glucose transporters determines the K_m. At present, we can only speculate that the kinetic parameters (K_m and V_{max}) were altered in GLUT4/2L in a way that their changes compensated each other at 1 mM glucose, and that only a minor difference to the GLUT4 resulted (V_{max} increase and K_m increase in GLUT4/2L). Since higher glucose concentrations interfere with the reconstitution assay, we could not analyze the possible V_{max} or K_m changes in our expression system.

Two of the constructs (GLUT2/4 and GLUT4/2) investigated here appeared completely inactive, because no additional glucose transport activity nor any ligand binding was detected in cells transfected with these constructs. Western blots indicated that degradation might be responsible for the inactivity of the GLUT2/4, but showed no abnormality of the GLUT4/2. Thus, we assume that the exchange of helix 1 introduced a structural alteration that affected the processing of the transporters in the COS-7 cells, and gave rise to premature proteolysis (GLUT2/4) or improper folding (GLUT4/2). This problem appeared to be specifically related to the expression of the constructs in COS-cells, because GLUT4/2 was capable of transporting glucose when expressed in *Xenopus* oocytes [15].

The finding that the C-terminal, cytoplasmatic tail (construct GLUT4/2T) is not important for glucose transport activity of the GLUT4 is in apparent contrast to a previous report [29]. In that study, replacement of the C-terminal tail of GLUT1 with that of the GLUT2 conferred a GLUT2-like glucose transport activity. Several possibilities have to be considered in order to explain this discrepancy. Firstly, it cannot be excluded that the role of the C-terminus for transport activity is different in GLUT1 and GLUT4. Secondly, it should be noted that the present data were obtained with a cell-free system, whereas those of Katagiri et al. [29] were from intact cells. Finally, it has to be stated that we have assayed the glucose transport activity at a single glucose concentration (1 mM), and that the alterations in K_m and V_{max} might have compensated each other at that concentration. However, our data clearly indicate that the C-terminal tail is not important for the high-affinity binding of cytochalasin B or forskolin to the GLUT4.

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