Exofacial Epitope-tagged Glucose Transporter Chimeras Reveal COOH-Terminal Sequences Governing Cellular Localization

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Abstract. The insulin-regulated adipocyte/skeletal muscle glucose transporter (GLUT4) displays a characteristic steady-state intracellular localization under basal conditions, whereas the erythrocyte/brain transporter isoform (GLUT1) distributes mostly to the cell surface. To identify possible structural elements in these transporter proteins that determine their cellular localization, GLUT1/GLUT4 chimera cDNA constructs that contain the hemagglutinin epitope YPYDVPDYA (HA) in their major exofacial loops were engineered. Binding of monoclonal anti–HA antibody to non-permeabilized COS-7 cells expressing HA-tagged transporter chimeras revealed that expression of transporters on the cell surface was strongly influenced by their cytoplasmic COOH-terminal domain. This method also revealed a less marked, but significant effect on cellular localization of amino acid residues between transporter exofacial and middle loops. The subcellular distribution of expressed chimeras was confirmed by immunofluorescence microscopy of permeabilized COS-7 cells. Thus, HA-tagged native GLUT4 was concentrated in the perinuclear region, whereas a chimera containing the COOH-terminal 29 residues of GLUT1 substituted onto GLUT4 distributed to the plasma membrane, as did native GLUT1. Furthermore, a chimera composed of GLUT1 with a GLUT4 COOH-terminal 30-residue substitution exhibited a predominantly intracellular localization. Similar data was obtained in CHO cells stably expressing these chimeras. Taken together, these results define the unique COOH-terminal cytoplasmic sequences of the GLUT1 and GLUT4 glucose transporters as important determinants of cellular localization in COS-7 and CHO cells.

GLUCOSE entry into eucaryotic cells is catalyzed by a group of glucose transporter protein isoforms that exhibit ∽50% sequence identity relative to each other (reviewed in Gould and Bell, 1990). Much of the structural similarity among these transporters resides in 12 putative transmembrane sequences. In contrast, the more hydrophilic putative cytoplasmic and extracellular domains contain sequences unique to each isoform. It is thought that these latter regions might define divergent kinetic properties of transport catalysis, unique regulatory mechanisms, or membrane targeting signals. Some of these transporter proteins are restricted to certain tissues, while others are widely distributed. For example, GLUT2 is present mostly in liver, kidney, small intestine, and β cells of the pancreas (Bell et al., 1990), whereas GLUT4 is found exclusively in the insulin-sensitive tissues muscle and fat (Birnbaum et al., 1989; James et al., 1989; Charron et al., 1989; Kaestner et al., 1989; Fukumoto et al., 1989). GLUT1 on the other hand is expressed to some extent in most if not all tissues. Thus, the divergent tissue distribution patterns of the glucose transporter isoforms reinforce the concept that important differences in function characterize these proteins.

The position of cellular glucose uptake as the first step in the important process of sugar metabolism suggests that the transporter proteins are highly regulated. Mechanisms of regulation appear to include control of transporter gene transcription (Sivitz et al., 1989), transporter protein degradation (Haspel et al., 1986), catalytic activity (Harrison et al., 1992), and cell surface localization (Cushman and Wardzala, 1980; Suzuki and Kono, 1980). Muscle and fat cells exhibit a particularly striking acute stimulation of glucose transport rates in response to the hormone insulin. This response is mostly due to the catalytic activity of GLUT4 transporter proteins which are rapidly redistributed by insulin action to the cell surface membrane from a predominantly intracellular localization in the basal state (Zorzano et al., 1989; Harrison et al., 1991). Evidence available suggests that the GLUT4 proteins in non-stimulated cells are mostly present in *trans*-Golgi (Slot et al., 1991) or other intracellular vesicular structures (Smith et al., 1991). GLUT1 transporters are also present in these cells, but at a much lower concentration. The GLUT1 transporter proteins are already present at the cell surface to a significant degree in the basal state, although their further concentration at the plasma membrane

is also elicited by insulin (Zorzano et al., 1989; Harrison et al., 1990a). Recent evidence suggests that the intrinsic catalytic activity of GLUT1 proteins on the cell surface is markedly suppressed upon differentiation of 3T3-L1 fibroblasts in culture to adipocytes (Harrison et al.,. 1991a; Czech et al., 1992), and that this can be released by insulin and other agents (Harrison et al., 1990b, 1991b). Taken together, these observations indicate that GLUT1 and GLUT4 may be the primary targets of different regulatory pathways.

Recent studies have attempted to define structural elements in the GLUT4 and GLUT1 transporter isoforms that determine their divergent steady-state cellular membrane distributions (Piper et al., 1992; Asano et al., 1992). These experiments have led to remarkably divergent results and conclusions among different laboratories. For example, Piper et al. (1992) have reported a highly significant effect of the NH_2-terminal region of GLUT4 in governing the intracellular disposition of chimera GLUT4/GLUT1 transporter proteins expressed in CHO cells. These results were obtained using EM and immunofluorescence microscopy to detect cellular localization of glucose transporter chimera constructs. In contrast, Asano et al. (1992) reported that the membrane distribution of GLUT1/GLUT4 chimera proteins expressed in CHO cells did not reflect the presence or absence of NH_2-terminal sequences, but rather coincided with two regions located on either side of the middle loop of the glucose transporter structure. An additive effect to cause intracellular localization of transporter proteins by these two regions of GLUT4 were noted when substituted into the GLUT1 transporter structure (Asano et al., 1992). At present, it is not possible to ascribe the differences in results of these experiments to obvious explanations.

The aim of the present studies was to develop novel methodology for assessing structural elements of the glucose transporter proteins that determine steady-state cell surface versus intracellular distribution. In particular, we sought a technique to rigorously establish the presence of transporters at the cell surface as an alternative to standard immunofluorescence microscopy. The results presented here demonstrate the effectiveness of a newly devised technique based on the insertion of a nine amino acid residue hemagglutinin (HA)[1] epitope in the exofacial loop of glucose transporter proteins. This is accomplished by engineering DNA encoding this epitope into native and GLUT1/GLUT4 chimera constructs, expressing the tagged chimera proteins in COS-7 or CHO cells, and monitoring the binding of high affinity anti–HA epitope antibody to these fixed, nonpermeabilized cells. Data obtained by this new method, as well as by the usual immunofluorescence microscopy techniques, reveal that the major determinant of glucose transporter localization to cell surface versus intracellular membranes resides in the unique COOH-terminal cytoplasmic sequences of the transporter proteins.

Materials and Methods

Cell Culture

COS-7 and CHO-K1 cells were obtained from American Type Culture Collection (Rockville, MD). Media, trypsin, antibiotics, and G418 were from GIBCO/BRL (Gaithersburg, MD) and FBS was purchased from Upstate

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1. *Abbreviation used in this paper*: HA, hemagglutinin.

Biotechnology, Inc. (Lake Placid, NY). COS-7 cells were maintained in DME with 10% FBS, 50 U/ml penicillin, and 50 μg/ml streptomycin in a 37°C humidified CO_2 incubator. The cells were subcultured before reaching confluence. CHO-K1 cell lines were maintained in Ham's F-12 medium with 10% FBS, 50 U/ml penicillin, and 50 μg/ml streptomycin in a 37°C humidified CO_2 incubator.

Construction of HA-tagged Chimera Transporters

The cloning of the HepG2 GLUT1 cDNA was described previously (Harrison et al. 1990b). The full-length cDNA for GLUT4 was cloned from a rat skeletal muscle cDNA library. All initial constructions were carried out in pUC18 or pUC19.

The construction of untagged chimeras was undertaken in a similar manner to the construction of chimeras 1(1-199)/4 and 1(1-462)/4 described previously (Pessino et al., 1990). Convenient existing restriction sites were used to switch domains between the two transporter isoforms whenever possible. When existing restriction sites could not be found, new ones were made by site-directed mutagenesis such that the amino acid sequence was not altered. All of the constructions were confirmed by sequencing through the changed areas. The HA tag was inserted in the NH_2 terminus or exofacial loop of GLUT1 and GLUT4 using oligonucleotides encoding the sequences detailed below and having convenient restriction sites on the ends such that the tag could be inserted at the desired position in the protein. When necessary, HA tag insertions were carried out in subcloned fragments of the cDNA and the full length regenerated after insertion of the tag was confirmed by sequencing. Once the tag was inserted in the exofacial loop of the wild type transporters, these domains were swapped appropriately with the corresponding domains of the untagged chimera transporters to generate exofacially tagged chimeras. All tagged chimera transporters were cloned into the expression vector pCMV.

GLUT1N has the sequence YPYDVPDYA inserted after the first methionine. GLUT4N has the sequence AYPYDVPDYA inserted after the first methionine. Exofacially tagged chimera transporters containing GLUT1 sequences in the NH_2 terminus have the sequence IDYPYDVPDYA inserted between amino acids 53 and 54, in the predicted first exofacial loop. Transporters with GLUT4 NH_2 termini have the sequence IDYPYDVPDYA inserted between amino acids 83 and 84. Domain switches were detailed in the legend of Fig. 1.

Transient Expression of HA–Epitope–tagged Chimera Transporter cDNAs in COS-7 Cells

COS-7 cells were seeded at 100,000 cells per 22-mm round, glass coverslip and transfection of HA epitope–tagged chimera transporter cDNAs was performed by the calcium phosphate precipitation method as described (Gorman, 1985) or by the use of the lipofection reagent DOTAP (Boehringer Mannheim Biochemicals) according to the recommended protocol. Cells were analyzed by immunofluorescence 48 h later.

Stable Expression of Chimera Transporter cDNAs in CHO-K1 Cells

Subconfluent CHO-K1 cells were co-transfected with pRSVneo and chimera transporter cDNAs by the calcium phosphate method described. G418-resistant colonies were picked up with the use of cloning cylinders and expanded. Positive cell lines were identified using immunofluorescence with anti-HA or anti-GLUT4 antibodies. Expression was confirmed by Western blotting of total cellular membranes.

Immunofluorescence of Transfected Cells

48 h after transfection, COS-7 cells were washed three times in PBS (171 mM NaCl, 10 mM Na_2HPO_4, 3.3 mM KCl, 1.8 mM KH_2PO_4), fixed for 10 min at room temperature in 4% formaldehyde in PBS, and rewashed three times in PBS. The fixed cells were then incubated with PBS containing 1% FBS and anti-HA antibody (mouse monoclonal 12CA5; BAbCO, Berkeley, CA) diluted 1:1,000, for 2–3 h at room temperature. The cells were washed and bound primary antibody was detected with FITC-coupled goat anti-mouse IgG for 30 min at room temperature. After washing, the cells were postfixed with 4% formaldehyde in PBS for 5 min at room temperature. Cells were then permeabilized by incubating with PBS containing 1% FBS and 0.5% Triton X-100 for 30 min at room temperature. Cells were then incubated with a 1:1,000 dilution of either rabbit anti–GLUT4 IgG (R1288) or monoclonal anti–HA antibody (12CA5) depending on the COOH-

terminal structure of the chimera for 18 h at 4°C. The cells were again washed, and bound primary antibodies detected with a 1:1,000 dilution of rhodamine-coupled goat anti–rabbit or anti-mouse IgG (Tago, Inc., Burlingame, CA). The cells were thoroughly washed and the coverglasses were mounted in 90% glycerol + 2.5% DABCO.

Samples were visualized on a microscope (IM-35; Carl Zeiss, Oberkochen, Germany), using a Nikon Apo 60/1.4 oil immersion lens. Images were recorded using a thermoelectrically cooled charged-couple device camera (Photometrics Ltd., Tucson, AZ). CHO-K1 cell lines were analyzed by immunofluorescence essentially as described above, except that the cells on coverslips were permeabilized directly after fixation and total cellular staining was detected with anti-HA antibody or anti-GLUT4 antibody (R1288) as necessary. Primary antibodies were detected with FITC-conjugated goat anti-mouse or goat anti-rabbit second antibodies as above.

Results

Exofacial HA Epitope-tagged GLUT1 Is Bound by Antibody 12CA5 in Intact Cells

As a means of assessing the concentrations of glucose transporters at the cell surface membrane, we devised a strategy to confer a known antibody recognition site on the predicted exofacial loops of these proteins. To achieve this objective, cDNA constructs encoding HA-tagged GLUT1, GLUT4, and the GLUT1/GLUT4 chimera transporters were prepared as depicted schematically in Fig. 1. DNA encoding the nine amino acid HA epitope sequence YPYDVPDYA was inserted into the GLUT1 exofacial loop or into the GLUT4 exofacial loop. Chimera GLUT1/GLUT4 cDNA constructs containing these exofacial loop HA inserts were then engineered as indicated. In addition, GLUT1 and GLUT4 constructs were prepared containing HA epitope tags at their NH_2 termini, which are predicted to extend into the cytoplasmic domain (GLUT1N and GLUT4N in Fig. 1). The cDNA constructs were then ligated into the expression vector pCMV and used for transient expression in COS-7 cells (see Figs. 2–5). Some of these constructs were also stably transfected into CHO cells (see Fig. 6). Insertion of the HA epitope into the GLUT1 structure at either the NH_2 terminus or exofacial loop did not disrupt function because CHO cells overexpressing these proteins exhibited the expected increase in glucose transport activity (data not shown). Previous data has also demonstrated that GLUT1/GLUT4 chimera constructs are functional as defined by ability to transport glucose (Pessino et al., 1991; Asano et al., 1992). Fig. 1 summarizes the cellular distributions observed with these expressed constructs, as described in detail below.

The HA-tagged glucose transporter proteins depicted in Fig. 1 were transiently expressed in COS-7 cells. The cell surface concentration of each construct was analyzed by immunofluorescence microscopy of non-permeabilized cells using a monoclonal anti–HA epitope antibody (12CA5) followed by a FITC-coupled anti–mouse secondary antibody. Subsequently, the cellular localization of all expressed transporters in the same cells was determined by permeabilization with 0.5% Triton X-100, a second exposure to the same primary antibody, and then incubation with a rhodamine-coupled anti–mouse secondary antibody. Fig. 2 shows the expected divergent cellular disposition of the NH_2-terminal (GLUT4N) and exofacial (GLUT4X) HA-tagged native GLUT4 vs. the NH_2-terminal (GLUT1N) and exofacial (GLUT1X) HA-tagged native GLUT1 in fixed, permeabilized COS-7 cells (top). GLUT4N and GLUT4X exhibit
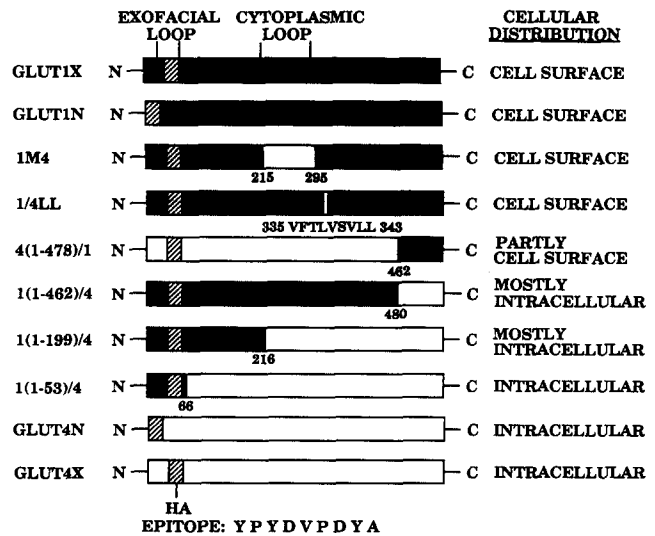

Figure 1. HA epitope-tagged chimeras and their comparison with GLUT1 and GLUT4 sequences. The location of the HA-tag is indicated by the hatched areas at the NH_2 terminus or in the exofacial loop. GLUT1N has the HA sequence YPYDVPDYA inserted immediately after the methionine start codon. GLUT4N has the sequence AYPYDVPDYA following the first methionine. HA tags in the exofacial loop of chimeras containing GLUT1 sequences in the NH_2 terminus have the tag IDYPYDVPDYA inserted after amino acid 53. Exofacial HA tags in chimeras containing GLUT4 NH_2 termini have the epitope IDYPYDVPDYA inserted after amino acid 83. The amino acids I and D are added to insert a unique ClaI restriction site to confirm orientation. Chimera 1M4 contains amino acid 1-199 of GLUT1, the amino acids 215-295 from GLUT4 corresponding to the middle loop, and GLUT1 amino acids 280-492. GLUT1/4LL contains the amino acid sequence of GLUT1 except that GLUT1 amino acids 319 to 327 have been replaced with amino acids 335-343 from GLUT4. Chimera 1(1-462)/4 replaces the last 29 amino acids of GLUT1 with the corresponding 30 amino acids of GLUT4. Chimera 1(1-199)/4 contains amino acids 1-199 of GLUT1 and amino acids 216-509 of GLUT4 which correspond to the rest of the molecule. Chimera 1(1-53)/4 contains the first 53 amino acids of GLUT1, the HA epitope and amino acids 66-509 of GLUT4.

a highly concentrated perinuclear localization, whereas GLUT1N and GLUT1X are readily detected at the cell border as well as in the perinuclear region. These patterns of distribution were identical to those observed with native, untagged GLUT4 and GLUT1, detected with their respective anti–COOH-terminal peptide antibodies, following expression in COS-7 cells (not shown).

To directly determine the levels of expressed transporter proteins on the cell surface, binding of anti-HA epitope antibody to non-permeabilized COS-7 cells was examined. As expected, exofacial HA epitope-tagged GLUT1X was readily detected under these non-permeabilized conditions (Fig. 2, bottom) in the same cells found to express this protein by labeling after permeabilization (Fig. 2, top). No signal was observed in non-permeabilized COS-7 cells expressing the GLUT4N, GLUT1N, or GLUT4X, as predicted by either the cytoplasmic disposition of the HA tag (GLUT4N and GLUT1N) or by the intracellular localization of the transporter proteins (GLUT4X). Taken together, these results confirm the utility of this experimental strategy using exofa-
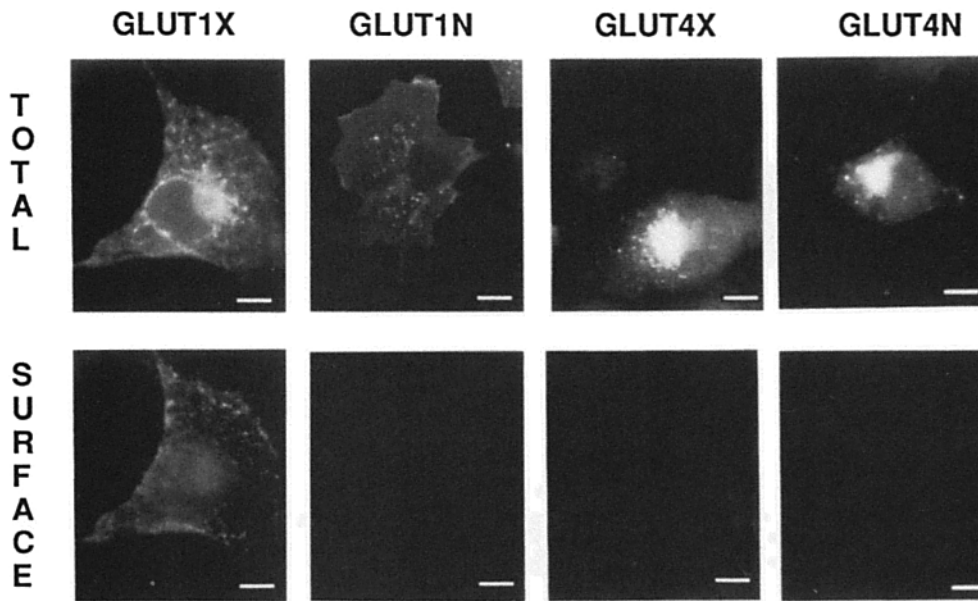
Figure 2. Immunofluorescence microscopy of HA-tagged GLUT1 and GLUT4 transporters transiently expressed in COS-7 cells. COS-7 cells transiently transfected with the indicated constructs were fixed in 4% formaldehyde 48 h after transfection. Cell surface transporters (*SURFACE*) were detected by incubation with anti-HA antibody (*12CA5*), and FITC-conjugated goat anti–mouse IgG before permeabilization. The cells were then permeabilized and total cellular transporters (*TOTAL*) were detected with anti-HA antibody and rhodamine-coupled goat anti-mouse (*GLUT1*) or anti-GLUT4 antibody (*R1288*) and rhodamine-coupled goat anti–rabbit antibodies (*GLUT4*). Bar, 10 μm.

cial HA epitope tagging to determine the cell surface localization of glucose transporters.

The Variable COOH-terminal Cytoplasmic Domain Is a Major Determinant of Glucose Transporter Localization

Using the above methodology, the extent to which extreme COOH-terminal sequences on the glucose transporters influenced cellular localization was evaluated. Continuing from the last COOH-terminal membrane spanning segment, the first 11 amino acid residues of the predicted COOH-terminal cytoplasmic tails of GLUT1 and GLUT4 are highly similar (~80% sequence identity), whereas the remaining COOH-terminal tail residues are quite dissimilar (~20% se-

quence identity). These divergent COOH-terminal residue segments on the GLUT1 and GLUT4 proteins have been switched in constructs 4(1-478)/1 and 1(1-462)/4 (see Fig. 1). In this series of experiments, the exofacial HA-tagged GLUT1X and GLUT4X also displayed the usual cell surface and perinuclear dispositions, respectively (Fig. 3). Remarkably, substituting the COOH-terminal 30 amino acids of GLUT4 onto GLUT1 (1[1-462]/4) caused a perinuclear localization of the chimera, as visualized in permeabilized cells (Fig. 3, *top*). When non-permeabilized COS-7 cells were analyzed with the 12CA5 antibody, GLUT1X-expressing cells were readily observed, whereas no signal above background was detected in cells expressing the 1(1-462)/4 glucose transporter construct (Fig. 3, *bottom*). Chimera 1(1-
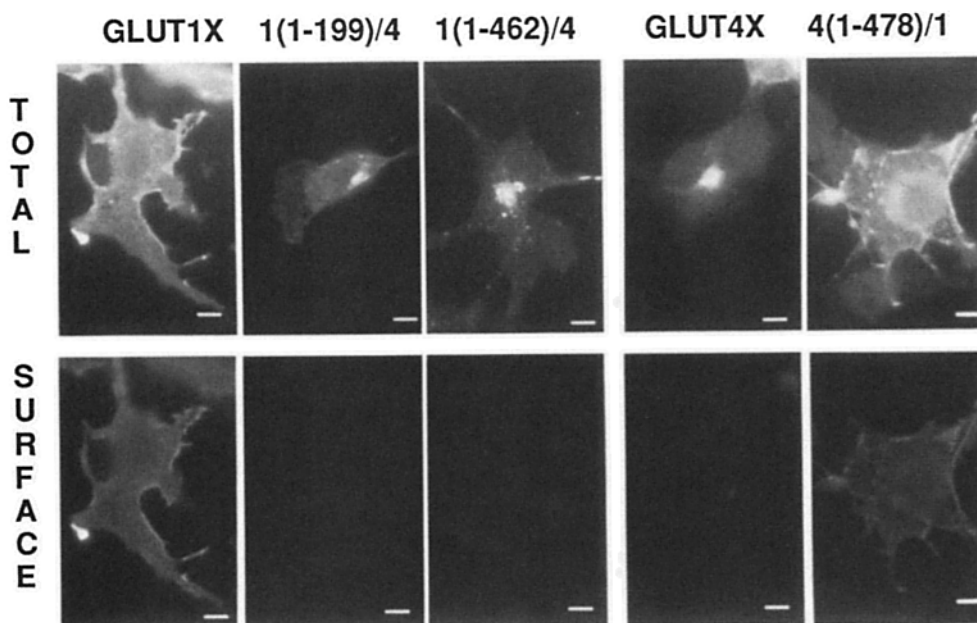

Figure 3. Immunofluorescence microscopy of HA-tagged GLUT1, GLUT4, and three transporter chimeras transiently expressed in COS-7 cells. COS-7 cells transiently transfected with the indicated constructs were fixed in 4% formaldehyde 48 h after transfection. Cell surface (*SURFACE*) and total (*TOTAL*) immunoreactivity was analyzed as described in the legend to Fig. 2. Bar, 10 μm.
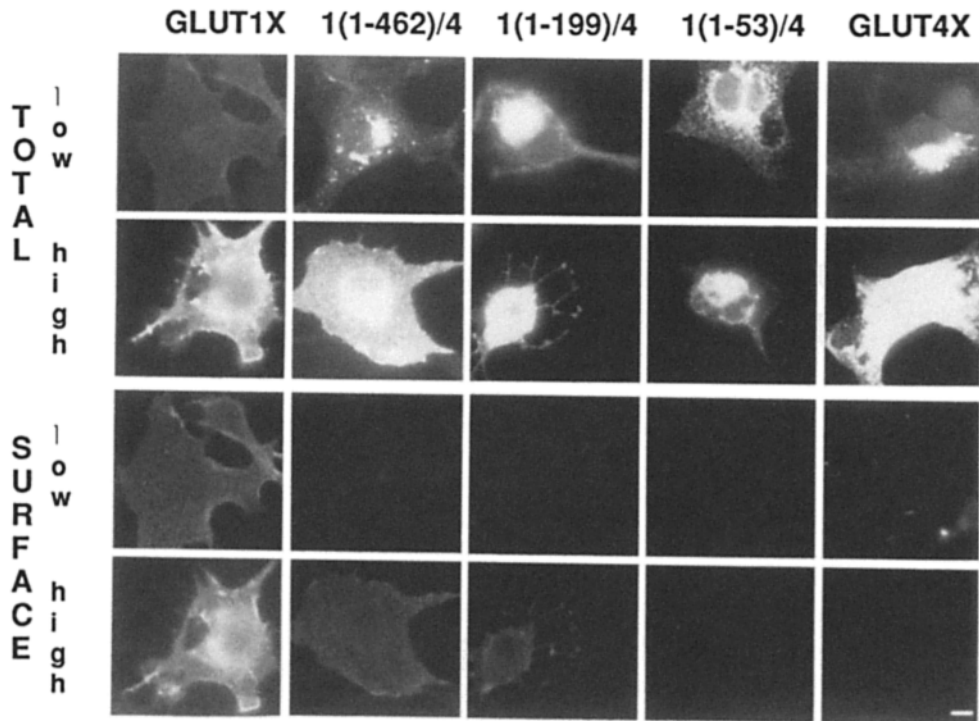
GLUT1X   1(1-462)/4   1(1-199)/4   1(1-53)/4   GLUT4X

T O T A L — low / high
S U R F A C E — low / high

Figure 4. Effect of expression level on cell surface localization of GLUT1, GLUT4, and three transporter chimeras transiently expressed in COS-7 cells. COS-7 cells transiently transfected with the indicated constructs were fixed in 4% formaldehyde 48 h after transfection. Cell surface (*SURFACE*) and total (*TOTAL*) immunoreactivity was analyzed as described in the legend to Fig. 2. Two different levels of expression of each construct were analyzed. Top panels of both galleries represent cells with low expression levels, whereas bottom panels in both galleries represent cells with higher expression levels. Bar, 10 μm.

199)/4 containing the NH₂-terminal 199 residues of GLUT1 and the remaining COOH-terminal residues of GLUT4 also exhibited an intracellular localization when expressed in COS-7 cells (Fig. 3).

A chimera in which the 29 COOH-terminal residues of GLUT1 were substituted onto GLUT4 (4[1-478]/1) confirmed the importance of the variable COOH-terminal sequences of GLUT1 and GLUT4 in determining whether these proteins distribute significantly to the plasma membrane. Thus, the 4(1-478)/1 protein was readily observed when analyzed in non-permeabilized COS-7 cells with the 12CA5 antibody (Fig. 3, *bottom*), and displayed an overall distribution pattern that resembled native GLUT1 when visualized in permeabilized cells (Fig. 3, *top*). Comparison of the intensity of cell surface staining of GLUT1X vs. 4(1-478)/1 at similar levels of total transporter expression indicated the former achieves a higher concentration at the cell surface. This suggests that other structural elements may also play a significant role in glucose transporter membrane distribution. Taken together, the data in Fig. 3 reveal a heretofore unrecognized major cell localization determinant in the COOH-terminal region of the GLUT1 and GLUT4 transporter proteins.

A Transporter Domain between the Exofacial and Extracellular Loops Influences Cellular Localization

The similar intracellular localization patterns of the 1(1-199)/4 and 1(1-462)/4 glucose transporter chimeras shown in Fig. 3 contrast sharply with the observations made by other laboratories. Asano et al. (1992) found a partial intracellular localization displayed by a chimera similar to our 1(1-199)/4, but extreme COOH-terminal cytoplasmic sequences did not influence transporter localization in their studies. Piper et al. (1992) reported a similar lack of effect of these latter se-

quences. We therefore tested whether the extent of total glucose transporter protein expression in COS-7 cells might influence the apparent cellular localization of transporters. The presently developed methodology allows us to rigorously compare both total expression levels of transporters and the concentration of these proteins on the cell surface.

Fig. 4 depicts the results of experiments in which the cell surface concentration of transporters was analyzed in COS-7 cells expressing either high or low amounts of total heterologously expressed transporter protein. Cells were chosen based on their immunofluorescence intensity after permeabilization and staining with 12CA5 and rhodamine-tagged anti-mouse Ig. The cell surface concentration of HA-tagged transporter was then determined by visualizing fluorescence from the FITC-labeled anti-Ig antibody, which reflects the anti-HA antibody bound to cells before permeabilization. COS-7 cells expressing low amounts of HA-tagged transporter protein showed completely intracellular distributions of GLUT4X and the three chimeras containing GLUT1 and sequences towards the COOH-terminal cytoplasmic tail of GLUT4 (1[1-462]/4, 1[1-199]/4, or 1[1-53]/4), as assessed by the lack of FITC staining. In contrast, cell surface GLUT1X was readily observed under these conditions (Fig. 4, panels *SURFACE, low*). The results confirm those of Fig. 3, and reinforce the lack of detectable influence of extreme NH₂-terminal sequences in governing intracellular localization (constructs 1[1-53]/4 vs. GLUT4X).

In contrast, similar analysis of COS-7 cells expressing high concentrations of these constructs revealed the presence of chimeras 1(1-462)/4 and 1(1-199)/4 on the cell surface, but not of 1(1-53)/4 or GLUT4X (Fig. 4, panels *SURFACE, high*). These results indicate that GLUT4 sequences 66-216 or the corresponding region of GLUT1, contain structural elements that may also play a role in the cellular localization of GLUT1 and GLUT4, in agreement with the conclusion of
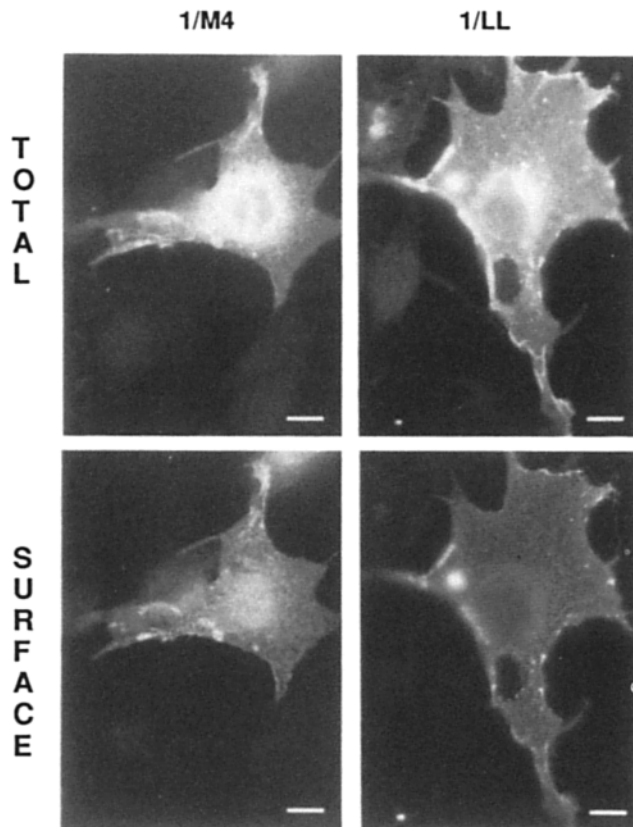
1/M4   1/LL

T
O
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S
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Figure 5. Immunofluorescence microscopy of transporter chimeras 1/M4 and 1/4LL transiently expressed in COS-7 cells. COS-7 cells transiently transfected with the indicated constructs were fixed in 4% formaldehyde 48 h after transfection, and analyzed as described in Fig. 2.

Asano et al. (1992). In these present experiments, the influence of these sequences only becomes evident at high levels of glucose transporter expression.

The analysis depicted in Fig. 4 also appears to confirm the finding of Asano et al. (1992) that the extreme NH₂-terminal residues of GLUT4 fail to effect membrane distribution of this transporter protein. Experiments were also undertaken to examine the possible influence of the major GLUT4 intracellular loop on cellular localization of glucose transporters. A GLUT1 construct (1/M4) with this GLUT4 middle loop substitution was expressed in COS-7 cells and found to distribute in a manner identical to native GLUT1 when probed with 12CA5 antibody following fixation and permeabilization of the cells (Fig. 5, panel *TOTAL*). Cell surface display of this chimera was confirmed by detection of intense fluorescence images when non-permeabilized COS-7 cells were analyzed (Fig. 5, panel *SURFACE*). Similar findings were made using a GLUT1 construct (see 1/4LL construct in Fig. 1) in which the sequence of the seventh intracellular loop of GLUT1 was substituted for that of GLUT4 (Fig. 5). These results indicate that the GLUT4 sequences present in the fifth and seventh intracellular loops do not appear to influence transporter localization.

GLUT4 COOH-terminal Domain Also Confers a Perinuclear Localization in CHO Cells

A plausible explanation for the differences between our

findings indicating a key role in membrane trafficking for the cytoplasmic COOH-terminal tail domain of transporter proteins and those of others is the use of different cell types. Neither of the previous reports using transporter chimeras described experiments with COS cells, while both Piper et al. (1992) and Asano et al. (1992) used CHO cells for analysis of transporter localization. Therefore, in the present studies CHO cells were stably transfected with native GLUT1 and GLUT4 constructs, GLUT1X and GLUT4N, and chimera 1(1-199)/4 depicted in Fig. 1. Stably transfected cell lines expressing a chimera 1(1-462)/4 containing GLUT1 sequences except for the 30 amino acid COOH-terminal GLUT4 domain substitution (as in Fig. 1, but without the HA tag) were also developed. Analysis of GLUT4N and chimera 1(1-199)/4 localization with 12CA5 antibody in the transfected CHO cells after fixation and permeabilization showed a marked perinuclear concentration (Fig. 6), similar to that observed for these transporters in COS-7 cells (Figs. 2-4). Interestingly, GLUT1X displayed a distribution pattern that was almost exclusively localized to the cell periphery, with very little intracellular staining in these CHO cells (Fig. 6). In contrast, the 1(1-462)/4 chimera composed of GLUT1 sequences except for the extreme 30-residue GLUT4 COOH-terminal substitution displayed an intracellular, perinuclear distribution. Interestingly, at high levels of expression, this transporter chimera was also detected at the cell surface. These results confirm the findings made with similar glucose transporter chimera proteins expressed in COS-7 cells (Figs. 2-4), and define the variable COOH-terminal domain of these transporters as a critical determinant of cellular localization.

Discussion

A New Methodology to Quantify Cell Surface Transporters

A major objective of the present studies was the development of methodology able to provide direct, unambiguous assessment of glucose transporter proteins at the cell surface membrane. Such a technique could be used to quantify cell surface localization of glucose transporters, calculate the specific catalytic activity of transporter proteins, and provide information on the underlying cellular mechanisms responsible for acute redistribution of transporters to the plasma membrane in response to insulin. Previous attempts to estimate cell surface glucose transporter proteins quantitatively have been met with several obstacles. For example, immunoelectron microscopy has been used successfully to document insulin-mediated increases in glucose transporter protein in the vicinity of the plasma membrane (Blok et al., 1988; Slot et al., 1991; Smith et al., 1991), but the resolution obtained does not permit rigorous conclusions about whether transporter proteins are actually exposed to the extracellular space. Furthermore, the possibility that insulin action changes the accessibility of antibodies directed against the COOH-terminal cytoplasmic tail of GLUT4 in such studies has been raised (Smith et al., 1991). These problems similarly complicate standard immunofluorescence microscopy techniques.

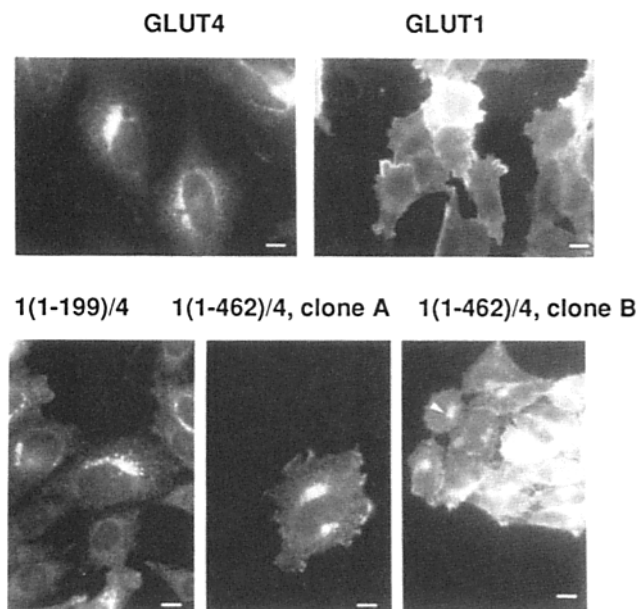Another method recently developed to detect exposed cell surface GLUT1 and GLUT4 involves affinity labeling the

GLUT4   GLUT1

1(1-199)/4   1(1-462)/4, clone A   1(1-462)/4, clone B

Figure 6. Immunofluorescence localization of GLUT1, GLUT4, and two transporter chimeras stably expressed in CHO cells. CHO-K1 cells stably expressing GLUT4N, GLUT1X, HA-tagged 1(1-199)/4, and two lines of untagged 1(1-462)/4 expressing the construct at low and high levels were grown on coverslips. The cells were fixed, permeabilized with PBS+1%FBS+0.5% Triton X-100, and expression detected with antibodies against HA (GLUT1, GLUT4, 1[1-199]/4) or against a COOH-terminal GLUT4 peptide (1(1-462)/4). Primary antibodies were detected with FITC conjugated anti–mouse or anti–rabbit antibodies. Images represented total staining within the cell. 1(1-462)/4 clone A is a low expressing cell line, 1(1-462)/4 clone B is a high expressing cell line. Arrowheads mark intracellular staining in 1(1-462)/4 clone B. Bar, 10 μm.

glucose transporters on intact cells with photoactive mannose (Holman et al., 1990) or glucose (Jhun et al., 1992) analogs. These agents react with the transporter proteins at glucose binding sites that operate as part of the transport catalysis mechanism. However, the use of these photolabeling agents for quantitative measurements may be inappropriate because they are based on the assumption that the properties of these sites do not change under different cellular conditions. Recent results suggest that in certain situations transporter glucose binding sites are inhibited or occluded by unknown cellular mechanisms (Harrison et al., 1992). Thus, alternative ways of assessing the concentration of cell surface glucose transporters are needed.

Insertion of the HA epitope into the predicted exofacial loops of GLUT1 and GLUT4 as a means of monitoring exposure of the tagged transporters to the extracellular space is documented by the present studies. This method provides unambiguous results, provided the surface membranes of cells analyzed are intact and impermeable to anti-HA immunoglobulin added to the medium. Thus, exofacial loop-tagged GLUT1X is intensely stained with antibody on the surface of transfected, non-permeabilized COS-7 cells, whereas GLUT-1 containing the HA tag on its cytoplasmic NH₂ terminus (GLUT1N) is not visualized under these conditions (Fig. 2). This latter construct serves as a valuable control for the possibility that processing the cells in any given experiment permeabilizes the plasma membrane to

anti-HA antibody. It should be noted that 12CA5 antibody binding to intact cultured cells can also be performed prior to fixation and analysis in order to assess cell surface transporters (data not shown). Other advantages of this technique include the high affinity of the commercially available 12CA5 antibody and the ability to assess transporter localization following transient transfection, when only a small percentage of cells express the desired transporter construct. Further, endogenous transporter proteins are not visualized in the assay because they lack the HA epitope. Thus, the new methodology is a highly specific and sensitive means to evaluate surface localization of specific transporter constructs in many different cell types.

The exofacial HA epitope-tagging method used here also reveals major limitations in the use of standard immunofluorescence microscopy for assessing cell surface proteins. Antibody staining in regions near the cell periphery can be easily mistaken for actual cell surface localization. For example, high levels of expression of chimera 1(1-53)/4 in permeabilized COS-7 cells causes intense staining with the 12CA5 antibody extending to areas along the plasma membrane (Fig. 4, panel *TOTAL, high*). However, in non-permeabilized COS-7 cells transfected with this construct, no cell surface binding to anti-HA antibody is detected. In contrast, 1(1-462)/4 transporter, which displays a similar staining pattern to 1(1-53)/4 in permeabilized COS-7 cells, is readily detected in non-permeabilized cells as well (Fig. 4). Thus, the HA-tagging method is able to document plasma membrane localization of transporter constructs even when this is difficult by the standard immunofluorescence microscopy techniques used by other investigators.

Glucose Transporter Domains Governing Cellular Localization

The present studies designed to assess actual exposure of glucose transporter chimeras on the cell surface have revealed the COOH terminus as a major structural determinant of transporter localization in COS-7 and CHO cells. This finding was unexpected because two independent laboratories have previously concluded that this domain is not involved in directing the cellular distribution of glucose transporters (Piper et al., 1992; Asano et al., 1992). It is all the more surprising because we were easily able to detect a striking effect using standard fluorescence microscopy by switching COOH termini on GLUT1 and GLUT4 chimera constructs. Detecting the HA epitope of the 4(1-478)/1 chimera on the cell surface of nonpermeabilized cells unequivocally confirmed the observations (Figs. 3 and 4). It is not clear why previous investigations failed to discover this effect. Both previous studies used CHO cells, as we did in the experiments depicted in Fig. 6. However, that levels of heterologous glucose transporter expression were much higher in the previous studies seems a likely possibility to consider. Piper et al. (1992) used a recombinant Sindbis viral vector to drive expression of glucose transporter chimeras in CHO-K1 cells to very high levels (equivalent to primary adipocyte content of GLUT4 after only 2 h of infection). Asano et al. (1992) used CHO-K1 cells exhibiting somewhat lower levels of heterologous transporter expression, and obtained consistent results in at least three cell clones for each chimera. Nevertheless, it is not clear from their data whether expression levels were similar to or higher than ours.

One of the criteria used by Asano et al. (1992) to assess cell surface concentration of glucose transporter chimeras was the increment in cellular deoxyglucose uptake over that of parental cells. These investigators observed high hexose uptake rates in CHO cells expressing a transporter chimera in which a COOH-terminal portion of GLUT4 was substituted onto GLUT1. In the present studies, stably transfected CHO cells expressing chimeras 1(1-199)/4 and 1(1-462)/4 also exhibited elevated hexose uptake rates, as we previously reported (Pessino et al., 1991), even though these chimeras exhibit a predominant perinuclear localization (Fig. 6). A plausible reason for this apparent paradox is that high levels of transporter expression saturate those mechanisms which prevent GLUT4 from accumulating at the cell surface. The marked contrast in staining pattern between native GLUT1, which localizes almost exclusively to the plasma membrane, vs. 1(1-199)/4 or 1(1-53)/4 which display an intense perinuclear presence, is striking in our experiments (Fig. 6). Nevertheless, at relatively high expression levels, significant amounts of these chimera proteins also accumulate on the cell surface, leading to increased deoxyglucose uptake. Taken together, the results from the previous studies and the present work suggest the following model: at physiological or lower levels of GLUT4 expression, the COOH-terminal cytoplasmic domain contains the major structural motif that determines intracellular localization. The influence of this motif saturates in this range of total transporter expression, whereas another structural element between the exofacial and intracellular loops has an effect that maintains the intracellular concentration of transporter even at higher levels of expression (e.g., Fig. 4).

Recent findings indicate that GLUT1 is present in intact cells, self-associated in oligomeric structures (Pessino et al., 1991). This is evidenced by co-immunoprecipitation of endogenous GLUT1 with chimera 1(1-462)/4 expressed in CHO cells using anti-COOH-terminal GLUT4 peptide antibody. Co-precipitation of native GLUT1 and native GLUT4 is not observed under similar conditions (Pessino et al., 1991). In purified form, the GLUT1 protein apparently forms dimers or tetramers (Hebert and Carruthers, 1991; Hebert and Carruthers, 1992). These observations potentially complicate interpretations on the behavior of expressed transporter chimeras due to their possible association with endogenous transporter proteins. Thus, it is possible that chimera trafficking patterns simply reflect their ability to oligomerize with and be directed by endogenous transporter proteins. However, it would appear that this problem does not apply to our major observation implicating the COOH terminus as a key determinant of cellular localization. Thus, the 1(1-462)/4 chimera displays an intracellular concentration (Fig. 3) despite the absence of endogenous GLUT4 in COS-7 or CHO cells. Furthermore, the 4(1-478)/1 chimera, present on the cell surface, does not contain the GLUT1 residues 1-199 that appear to be necessary for oligomerization with endogenous GLUT1 (Pessino et al., 1991). It is also unlikely that the purported influence of residues between the major exofacial and cytoplasmic loops in cellular distribution is related to oligomerization of 1(1-199)/4 with endogenous GLUT1 (Fig. 4). This is because this chimera displays predominantly intracellular pattern even at expression levels that are much higher than the endogenous GLUT1 protein.

The COOH-terminal cytoplasmic domains of GLUT4 and

GLUT1 could operate to confer the characteristic cellular distributions of these proteins through several types of mechanisms. One possibility is that the COOH terminus of GLUT1 contains information that drives its movement to the cell surface. Alternatively, the COOH-terminal domain of GLUT4 may contain information that confers its intracellular localization. This latter possibility is favored by substantial evidence indicating that movement of proteins through the secretory pathway to the plasma membrane occurs by default. According to this concept, no specific structural signals are required for the progression of proteins to the plasma membrane. In contrast, proteins that do not reach the cell surface immediately after synthesis (i.e., GLUT4) usually have specific structural elements that target the protein to a functionally relevant compartment. Signals in the COOH-terminal domain of GLUT4 could determine its intracellular localization by several conceivable mechanisms. For example, the signal could direct the transporter to a specialized intracellular vesicle following its synthesis and processing. Alternatively, the transporter might first reach the plasma membrane, and then be directed by an endocytosis pathway to a particular intracellular membrane pool. Interestingly, this mechanism appears to be involved in the synthesis of small synaptic vesicles in PC12 cells (Regnier-Vigouroux et al., 1991).

Analysis of the primary amino acid sequence of the GLUT4 COOH terminus reveals features that potentially contain targeting information. Residues 489 and 490 of GLUT4 are leucines, resembling dileucine motifs recently found to be important in the targeting of T cell receptor CD3 chains (Letourneur and Klausner, 1992) and in the sorting of the IGF-II/mannose-6-phosphate receptor to a prelysosomal/lysosomal pathway (Johnson and Kornfeld, 1992). Interestingly, the dileucine motif alone appears to efficiently target proteins to the endocytic pathway. Fig. 5 shows that a dileucine motif near the major intracellular loop has no detectable influence on glucose transport localization, and future work is devoted to similar evaluation of the COOH-terminal dileucine. Sequences contained between residues 53 and 199 of GLUT4 also appear to contribute to its intracellular localization. It is interesting that even at high levels of expression, chimeras containing such sequences are not expressed at the cell surface (Fig. 4). The additive effects of the 53-199 and the COOH-terminal regions suggest the existence of at least two independent retention/internalization determinants in GLUT4. An important future goal is to further define the structural nature of each determinant, and to explore whether the actions of insulin on the distribution of GLUT4 are exerted through the interactions of these determinants with the trafficking machinery of insulin-sensitive cells.

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