Identification of the Carboxy Terminus As Important for the Isoform-specific Subcellular Targeting of Glucose Transporter Proteins

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Abstract. Differential trafficking of glucose transporters contributes significantly to the establishment of a cell's capacity for hormone-regulatable hexose uptake. In the true insulin-sensitive peripheral target tissues, muscle and adipose, the transporter isoform GLUT1 resides on the cell surface and interior of the cell whereas the highly homologous isoform GLUT4 displays virtually exclusive intracellular sequestration, allowing the latter to redistribute to the cell surface in response to hormone. These patterns are equally pronounced in cells into which the transporters have been introduced by DNA-mediated gene transfer, suggesting that signals for isoform-specific sorting are recognized in diverse cell types. To determine the primary sequences responsible for the characteristic distributions, chimeric transporters were constructed in which reciprocal domains were exchanged between GLUT1 and GLUT4. In addition, a non-disruptive, species-specific epitope "tag" was introduced into a neutral region of the transporter to allow analysis of reciprocal chimeras using a single antibody. These recombinant transporters were stably expressed in HIH 3T3 and PC12 cells by retrovirus-mediated gene transfer, and were localized by indirect immunofluorescence and laser scanning confocal microscopy, as well as by staining of plasma membrane sheets prepared from these cells. The results indicate that the carboxy-terminal 30 amino acids are primarily responsible for the differential targeting of the glucose transporter isoforms GLUT1 and GLUT4, though there is a lesser additional contribution by the amino-terminal 183 amino acids.

EXPOSURE of muscle and adipose tissue to insulin stimulates a rapid and dramatic increase in glucose uptake, primarily by altering the subcellular distribution of glucose transporter proteins (for reviews see reference 4). At least two of the five hexose carrier isoforms identified to date are expressed in these cells and, although they were highly homologous in their primary and predicted secondary structures, are targeted to distinct subcellular locations (3, 19). In the basal state, the widely expressed GLUT1 is distributed to both the plasma membrane and the interior of the cell, whereas GLUT4, the transporter expressed exclusively in true insulin-responsive cell types, is sequestered in an intracellular storage vesicle (4). This observation suggests that differences intrinsic to the GLUT1 and GLUT4 proteins dictate their differential sorting. Thus, the current translocation model to describe the insulin-induced increase in glucose uptake in fat and muscle cells proposes that insulin evokes a redistribution of GLUT4, and to a lesser extent GLUT1, from the interior of the cell to the plasma membrane, thereby increasing the glucose transport capacity of the cell. This selective enrichment of GLUT4 at the cell surface is reversible, as removal of the hormone returns this isoform to its storage site in the interior of the cell (28, 31).

Support for such a translocation model derives from several studies, including the demonstration by immunoelectron microscopy (immunoEM) that GLUT4 localized to tubulovesicular elements of the TGN (11) and endosomes of rat brown adipose tissue shifts to the plasma membrane upon treatment with insulin (28). These findings have been confirmed by similar studies in isolated rat adipocytes, rat skeletal muscle, and rat cardiac muscle (25, 27, 29). Surface labeling of rat adipocytes with a photoactive hexose analog has allowed quantification of this redistribution, with insulin stimulation increasing GLUT1 labeling of the plasma membrane fivefold and GLUT4 15–20-fold (15). In mouse 3T3-L1-cultured adipocytes, as in rat fat cells, GLUT1 and GLUT4 display distinct staining patterns and differences in magnitude of hormone-dependent recruitment (6, 22).

Several lines of evidence support the proposition that the primary structures of GLUT1 and GLUT4 contain the information necessary for their differential subcellular targeting. When cDNAs were introduced into minimally insulin-responsive cell lines, the transporters were found in distinct subcellular locales: predominantly perinuclear for GLUT4 and cell surface for GLUT1. The two isoforms have been expressed in mouse 3T3-L1 and NIH 3T3 fibroblasts (12, 17), hamster CHO cells (23, 26), human HepG2 cells (12), and

rat PC12 cells (18). Even in such diverse cell types, each isoform shows a characteristic pattern of localization. These studies suggest that GLUT1 and GLUT4 are recognized in distinct ways by the trafficking pathways of the cell and that the machinery necessary to sequester GLUT4 in an intracellular pool is present in disparate cell types. Whether GLUT4 resides in the same organelle in insulin-responsive and nonresponsive cells is unclear, as are the mechanisms involved in sorting GLUT4 to this ill-defined compartment. Nonetheless, these results demonstrate that the sequences of these transporters must contain information capable of directing them to different cellular locales.

In an effort to identify the primary sequence(s) responsible for sorting GLUT1 and GLUT4 to different locations within the cell, we constructed chimeric transporters in which reciprocal domains were exchanged between the two isoforms. These recombinant transporters were expressed in identical cellular contexts and their localization analyzed. Piper et al. (23) and Asano et al. (2) have recently published analogous studies aimed at identifying the targeting sequence of GLUT4, but obtained conflicting results. We approached this problem by using stable expression in two cell lines of immunologically "tagged" chimeras, and concluded that both the amino and carboxy domains contribute to the targeting of the transporters, with the latter predominant.

Materials and Methods

DNA Constructs

The retroviral expression vector pDO-R (renamed pDOJ, a gift of Dr. Connie Cepko, Harvard Medical School, Boston, MA) has been described previously, as have the plasmids pDOJ-GT (the rat GLUT1 cDNA inserted into DOJ) and pDOJ-SM (the rat GLUT4 cDNA inserted into DOJ) (17). Chimeric transporters were constructed by fusing portions of the GLUT1 and GLUT4 cDNAs at common restriction sites either present in the wild-type sequence or engineered by polymerase chain reaction-based mutagenesis (13, 14, 30). All sequences produced by the polymerase chain reaction were confirmed using the Sequenase polymerase (United States Biochemical Corp., Cleveland, OH) according to the manufacturer's instructions. The nucleotide and amino acid numberings used below are according to published sequences of rat GLUT1 and GLUT4 (3, 5).

The general strategy for the construction of chimeric transporters is summarized in Fig. 1. A BsmI site (see Fig. 1 *M*) present in the GLUT4 cDNA at nucleotide 653 (amino acid 183) was introduced into the GLUT1 cDNA at nucleotide 707 (amino acid 167) by changing nucleotide G708 to C and nucleotide C711 to T. The HindIII site (see Fig. 1 *D*) at nucleotide 895 (amino acid 230) in the GLUT1 cDNA was engineered into the GLUT4 cDNA (nucleotide 841, amino acid 246) by changing the following nucleotides: C841 to A, G842 to A, C843 to G, and G846 to T. The ApaI site (see Fig. 1 *A*) at nucleotide 1403 (amino acid 399) of the GLUT1 cDNA was introduced into the GLUT4 cDNA (amino acid 415) by changing nucleotide C1347 to G. The BglII site (see Fig. 1 *B*) present in the GLUT4 cDNA at nucleotide 1538 (amino acid 478) was added to the GLUT1 cDNA (amino acid 462) by changing nucleotide G1597 to T. The human epitope (see Fig. 1 *H*) was engineered into the rat GLUT1 cDNA by changing amino acid 239 from Arg to His (nucleotides C921 to T, G923 to A, and A924 to T). The naming of the chimeras and the amino acid contributions of GLUT1 and GLUT4 to each are shown in Fig. 1. In addition, all constructs bearing the human GLUT1 epitope also contained the identical 3′ untranslated region (3′-UTR)[1] contributed by the rat GLUT1 cDNA, independent of the source of the carboxy-terminal coding sequences. This was accomplished by overlapping polymerase chain reaction, in which the GLUT1 3′-UTR was added immediately after the GLUT4 termination codon (14, 16).

1. *Abbreviations used in this paper*: PM, plasma membrane; 3′-UTR, 3′-untranslated region; WGA, wheat germ agglutinin.

Cell Culture and Gene Transfer

NIH 3T3 and Psi2 mouse fibroblasts (21) were grown in DME supplemented with 10% calf serum whereas HeLa cells were maintained in DME plus 10% FBS. The rat pheochromocytoma cell line PC12 (10) was grown in DME containing 10% horse serum and 5% FBS on tissue culture plates coated with rat tail collagen (Biomedical Technologies, Stoughton, MA). Cells transfected or infected with retrovirus containing the *neo* resistance gene were maintained in medium supplemented with 200 μg/ml active concentration Geneticin (G418; GIBCO-BRL, Gaithersburg, MD). Retrovirus was harvested from pools of stably transfected Psi2 cells as described previously (17) and used to infect NIH 3T3 and PC12 cells. Colonies of G418-resistant cells were identified 10–14 d after retrovirus infection and were expanded singularly as clones or together as pools in 200 μg/ml G418.

Supernatant from hybridoma cells producing mAbG3 (1; a gift of Dr. Gus Lienhard, Dartmouth Medical School, Hanover, NH), a mAb specific for human GLUT1, was used undiluted for immunofluorescence or diluted 1:2 with 1% low-fat dried milk powder (Carnation Co., Los Angeles, CA) for Western blotting.

Analysis of Proteins

Total cellular membranes were prepared and subjected to SDS-PAGE and Western blot analysis as described (17) except that 1% low-fat dried milk powder was used as a blocking agent. Blots were quantitated using a Molecular Dynamics (Sunnyvale, CA) phosphorimager equipped with ImageQuant software.

NIH 3T3 cells plated on glass coverslips 2 d before an experiment were washed with PBS and fixed in 3% paraformaldehyde for immunofluorescence using rabbit polyclonal antisera directed against the carboxy terminus of GLUT1 or GLUT4 (17). For some experiments, anisomycin (100 μM) or cycloheximide (20 μg/ml) was present for 0–4 h before fixation. PC12 cells were plated on poly-D-lysine-coated coverslips and the following day 10 ng/ml NGF (purchased from the Director of Research at the University of Michigan, Flint, MI) was added to the medium. After 2 d, the cells were fixed with 3% paraformaldehyde and processed for immunofluorescence. All coverslips were mounted in Mowiol and the cells visualized with a Zeiss Axiophot fluorescence microscope (Carl Zeiss, Oberkochen, Germany) or a scanning laser confocal microscope (MRC600; BioRad Laboratories, Cambridge, MA), as indicated.

Preparation and analysis of plasma membrane (PM) sheets was performed as described previously (9, 23, 24). Briefly, cells were grown on poly-D-lysine-coated coverslips in 35 mm wells for 2 d or until confluent. The cells were washed twice with ice-cold buffer A (50 mM Hepes, pH 7.2, 100 mM NaCl) and twice with ice-cold buffer B (20 mM Hepes, pH 7.2, 100 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 μM leupeptin, 2 μg/ml trypsin inhibitor, 0.5 mM benzamidine). Each well was sonified in ice-cold buffer B for 3 s using a Branson 250 Sonifier (Branson Ultrasonics Corp., Danbury, CT) with a 0.8-mm tapered microtip placed 1 cm above the coverslip. The adherent PM sheets were then washed twice with ice-cold buffer B, fixed with 3% paraformaldehyde and processed for immunofluorescence as above, except without detergent. After incubating the coverslips with mAbG3 and rhodamine-conjugated goat anti-mouse secondary antibody (Tago, Inc., Burlingame, CA), the sheets were labeled for 15 min with 0.2 μg/ml FITC-conjugated wheat germ agglutinin (FITC-WGA; Vector Laboratories Inc., Burlingame, CA).

Results

Analysis of Group I Chimeric Transporters

As an initial approach, we divided the transporters into four domains based on convenient unique restriction sites which were present in one transporter and were engineered at the corresponding location into the cDNA encoding the other isoform. These first sites chosen were BsmI (M) located at sequences encoding membrane spanning region 5 (MS5), HindIII (D) in the large intracellular loop, and ApaI (A) between MS10 and MS11 (Fig. 1, Group I). Chimeric cDNAs constructed by conventional subcloning techniques were inserted into a retroviral expression vector and the viral supernatant was used to infect two different cell lines, mouse NIH

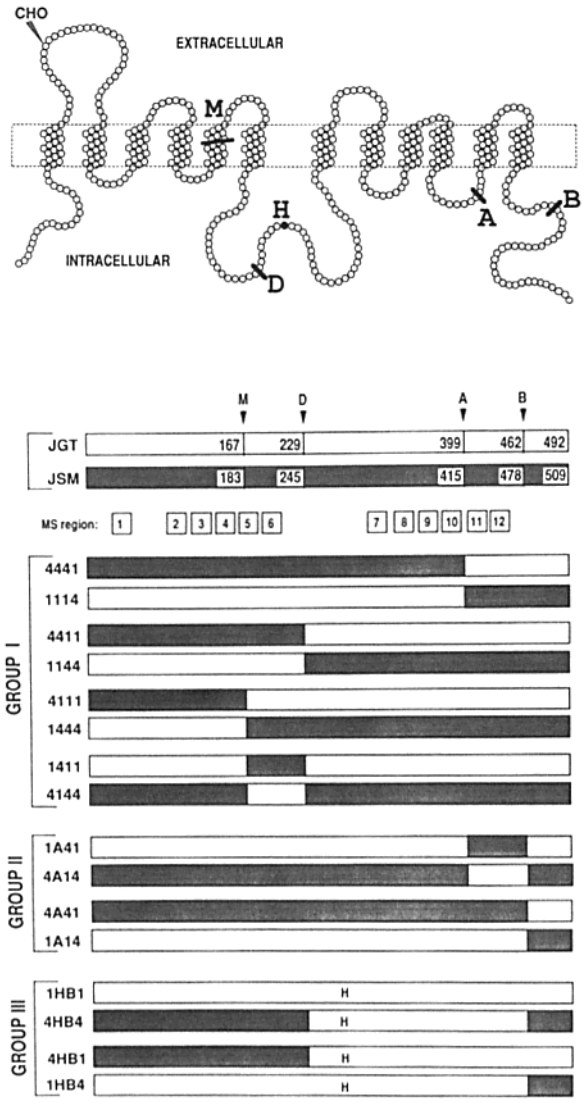

Figure 1. Structures of chimeric transporters. Predicted transmembrane topology of the glucose transporters (*top*). The indicated restriction sites BsmI (*M*), HindIII (*D*), ApaI (*A*), and BglII (*B*) were used to exchange portions of the GLUT1 and GLUT4 cDNAs. H designates the amino acid of GLUT1 altered to create the human epitope "tag" (see text). The contribution of GLUT1 (□) and GLUT4 (▩) to each chimera is shown schematically in the bottom half of the figure. Group I chimeras were constructed by exchanging segments of the transporters at sites M, D, and A and were named by a four digit code corresponding to the contribution of each isoform to the four domains. In Group II, the chimeras were named according to the contribution of GLUT1 or GLUT4 to the segments before and after the ApaI site (*A*). For the naming of Group III chimeras, H designates the human epitopes and B represents the BlgII site. Chimeras of Groups I and II contain the 3′-UTR of the isoform contributing the carboxy-terminal 30 amino acids whereas the chimeras in Group III all contain the GLUT1 3′-UTR. *MS*, membrane spanning.

3T3 and rat PC12 cells. Stable G418–resistant clones of each construct were expanded (NIH 3T3 cells) or pooled and differentiated with NGF (PC12 cells) and analyzed by indirect immunofluorescence microscopy using anti-peptide antibodies reactive with the carboxy terminus of the chimeras. A representative clone of each construct in NIH 3T3 cells is shown in Fig. 2; reciprocal chimeras are shown adjacent to each other. Wild-type GLUT1 (JGT) was found in a peripheral distribution in both cell types, consistent with cell surface expression (Fig. 2, *A* and *B*), whereas GLUT4 (JSM) was localized to the perinuclear region in NIH3T3 cells (Fig. 2 *C*) and to the perinuclear region of the cell body and the distal portions of the neuronal processes in PC12 cells (Fig. 2 *D*), as has been previously reported (18). Indirect immunofluorescence of cells expressing the chimeric transporters 4441, 4411, and 4111 with α-GLUT1 displayed staining of both the perinuclear region and the cell surface, as well as some staining in the termini of the neurites in PC12 cells (Fig. 2, *E*, *F*, *I*, *J*, *M*, and *N*). These results indicate that the amino-terminal third of GLUT4 is capable of diverting some of GLUT1 from the plasma membrane to an intracellular site. Localization of the reciprocals of these chimeras (1114, 1144, and 1444) with α-GLUT4 showed predominant staining in the perinuclear region of both cell types, and in the ends of the neuronal processes in PC12 cells, with minimal labeling of the plasma membrane (Fig. 2, *G*, *H*, *K*, *L*, *O*, and *P*). When the amino- and the carboxy-termini were contributed by the same isoform, i.e., chimeras 1411 and 4144, the apparent subcellular distribution was indistinguishable from that of wild-type GLUT1 and GLUT4, respectively, in both NIH 3T3 and PC12 cells (Fig. 2, *Q–T*).

Group II Chimeric Transporters Further Delineate the Carboxy-terminal Signal

The above data suggest that the carboxy-terminal region is important for the differential sorting of GLUT1 and GLUT4 in at least two distinct cell types. To further resolve the location of relevant sequences in this region of the transporter, a BglII site (Fig. 1, *B*) located in sequences encoding the amino acid 31 residues from the end of GLUT4 was engineered into the corresponding position of the GLUT1 cDNA. Using this site, as well as the ApaI site (*A*) following MS10, chimeras were constructed in which either only the last two MS regions or only the cytoplasmic carboxy terminus of each transporter was replaced by the corresponding domain of the other isoform (Fig. 1, Group II). The cDNAs were introduced into NIH 3T3 cells via retroviral infection and stable G418-resistant clones were analyzed by indirect immunofluorescence microscopy. Two representative clones of each chimera are shown in Fig. 3. The substitution of MS11 and MS12 of GLUT1 by that of GLUT4 did not alter its localization to the cell surface (Fig. 3, *A* and *B*; chimera *1A4I*), and the replacement of this region of GLUT4 by that of GLUT1 did not affect its perinuclear distribution (Fig. 3, *C* and *D*; chimera *4A14*). However, when the carboxy-terminal 30 amino acids of GLUT1 were replaced by the corresponding domain of GLUT4, the chimera was localized predominantly in a perinuclear distribution similar to that of GLUT4 (Fig. 3, *G* and *H*; chimera *1A14*). The reciprocal chimera, 4A41, displayed an "intermediate" phenotype, in which the transporter showed staining at the cell surface as well as intracellularly (Fig. 3, *E* and *F*).

Introduction of a Species-specific Epitope Tag

The experiments described thus far appeared to identify a prominent determinant of subcellular trafficking in the carboxy terminus of the transporters. Since this region of the

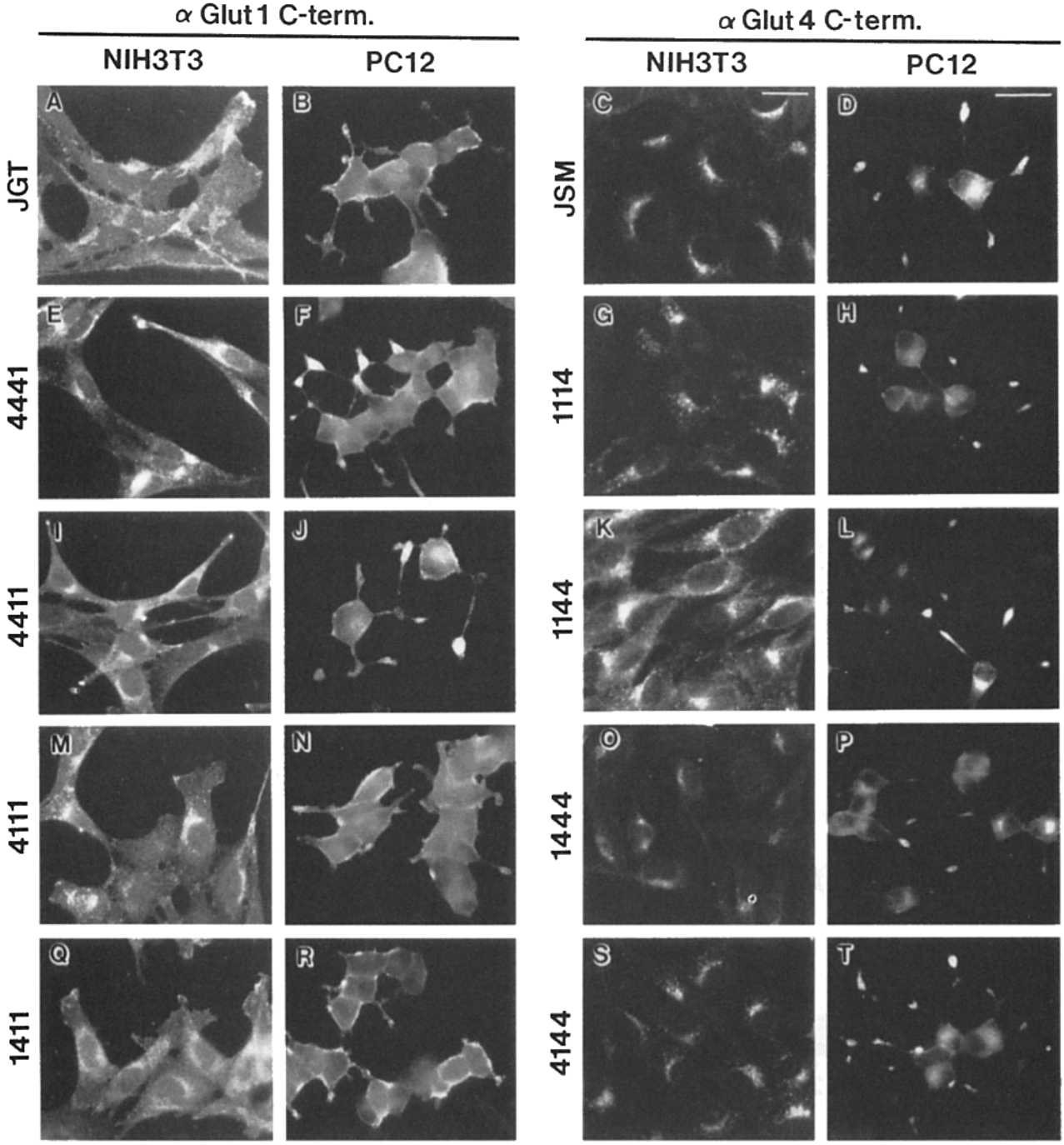

Figure 2. Indirect immunofluorescence of group I chimeric transporters in NIH 3T3 and PC12 cells. Clones of NIH 3T3 cells and pools of NGF-treated PC12 cells stably infected with retrovirus containing GLUT1 (*JGT*), GLUT4 (*JSM*), or the indicated chimeric cDNAs were fixed, permeabilized, and stained with an antibody to the GLUT1 carboxy terminus (*A*, *B*, *E*, *F*, *I*, *J*, *M*, *N*, *Q*, and *R*) or an antibody to the GLUT4 carboxy terminus (*C*, *D*, *G*, *H*, *K*, *L*, *O*, *P*, *S*, and *T*). For each antibody, the same photographic and printing conditions were used for all chimeras. Bar, 20 μM.

protein is the same as that recognized by the antibodies, we were wary of artifacts resulting from the inability to analyze reciprocal chimeras with identical immunological reagents. Thus, we elected to "tag" the chimeras with a neutral epitope. As shown in Figs. 2 and 3, the exchange of sequences in the middle portion of the transporters, including the large cytoplasmic loop, had no effect on their subcellular distributions. A mAb has been raised against the human GLUT1 transporter (mAbG3) which recognizes amino acids 231–246 in this central loop of the human GLUT1 protein, but does not react with the rat GLUT1 protein (1, 8). These species differ by only a single amino acid in this region. We therefore changed residue 239 in the rat GLUT1 cDNA from Arg to His, as it occurs in the human transporter, naming the mutant 1HB1 (Fig. 1). Immunofluorescence microscopy confirmed that mAbG3 recognized the glucose transporter endogenous to the human cell line HeLa (Fig. 4 *A*), but it did not detect either the endogenous transporter present in

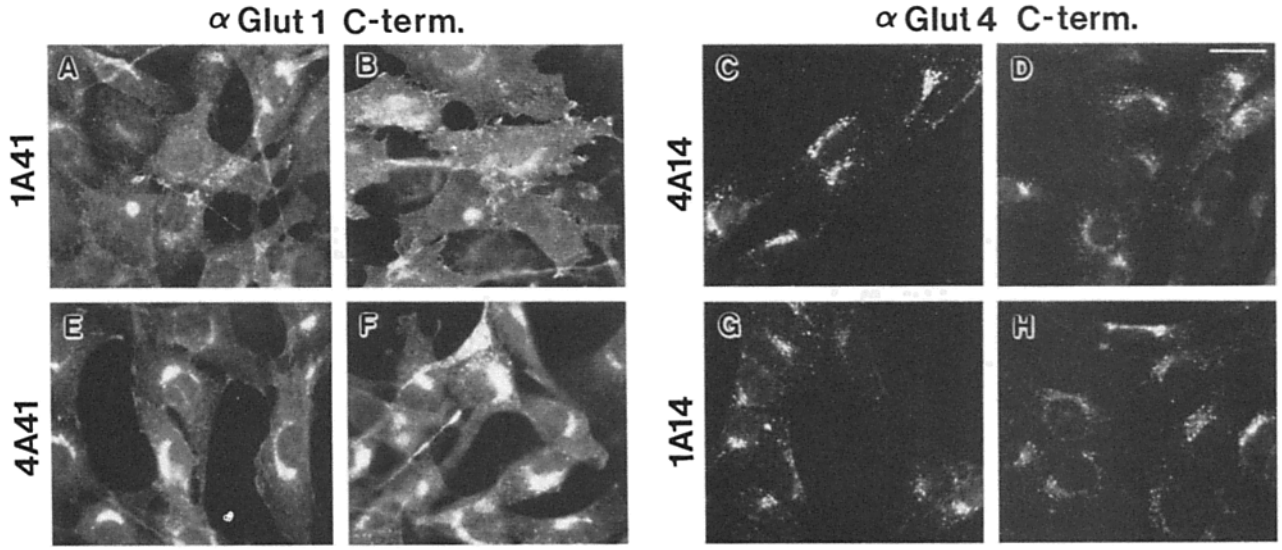

Figure 3. Indirect immunofluorescence of group II chimeric transporters in NIH3T3 cells. Clones of NIH 3T3 cells stably infected with retrovirus containing the indicated chimeric cDNAs were fixed, permeabilized and stained with an antibody to the GLUT1 carboxy terminus (*A*, *B*, *E*, and *F*) or an antibody to the GLUT4 carboxy terminus (*C*, *D*, *G*, and *H*). For each antibody, the same photographic and printing conditions were used for all chimeras. Bar, 20 μM.

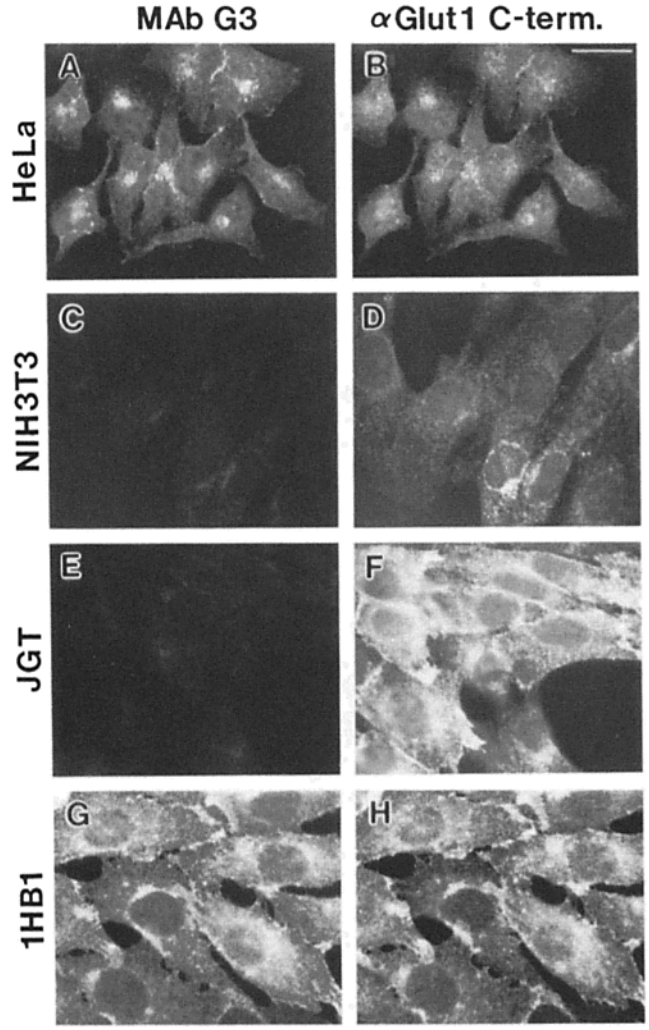

Figure 4. Double label indirect immunofluorescence shows the specificity of mAbG3 and the human epitope "tag." HeLa cells (*A* and *B*), untransfected NIH 3T3 cells (*C* and *D*), NIH 3T3 cells expressing rat GLUT1 (*JGT*; *E* and *F*) and NIH 3T3 cells expressing the "tagged' chimera 1HB1 (*G* and *H*) were fixed, permeabilized, and stained for double immunofluorescence with mAbG3 and rhodamine-conjugated goat anti-mouse antibody (*A*, *C*, *E*, and *G*) and with an antibody to the GLUT1 carboxy terminus and FITC-conjugated goat anti-rabbit antibody (*B*, *D*, *F*, and *H*). For each antibody, the same photographic and printing conditions were used for all cell lines. Bar, 20 μM.

mouse NIH 3T3 cells (Fig. 4 *C*) or the rat GLUT1 protein expressed in NIH 3T3 cells (Fig. 4 *E*, *JGT*). However, the antibody did recognize the rat GLUT1 protein in which the human epitope had been inserted, as assayed after introduction of the 1HB1 transporter into NIH 3T3 cells (Fig. 4 *G*). For comparison, the same cells were labeled with the anti-peptide antibody directed against the carboxy terminus of GLUT1. This antisera recognized the GLUT1 protein regardless of species: the human transporter in HeLa cells (Fig. 4 *B*), the mouse GLUT1 present in NIH 3T3 cells (Fig. 4 *D*), and the overexpressed rat GLUT1 without (Fig. 4 *F*, *JGT*) and with (Fig. 4 *H*, 1HB1) the human epitope. Thus, this single amino acid change in the rat GLUT1 sequence allowed detection of the transporter at a site not implicated in sorting of the isoforms.

Analysis of the Group III Chimeric Transporters Containing the Epitope Tag

Chimeric transporters were constructed in which the central portions were contributed by the 1HB1 mutant and the ends represented all possible combinations of GLUT1 and GLUT4 (Fig. 1, Group III). In addition, to reduce potential differences in levels of expression due to variations in mRNA stability, these constructs were designed to contain an identical 3'-UTR contributed by the rat GLUT1 cDNA. At least eight clones of NIH 3T3 cells infected with retrovirus encoding each of the "tagged" chimeras were expanded and the levels of transporter determined by Western blotting of total cellu-

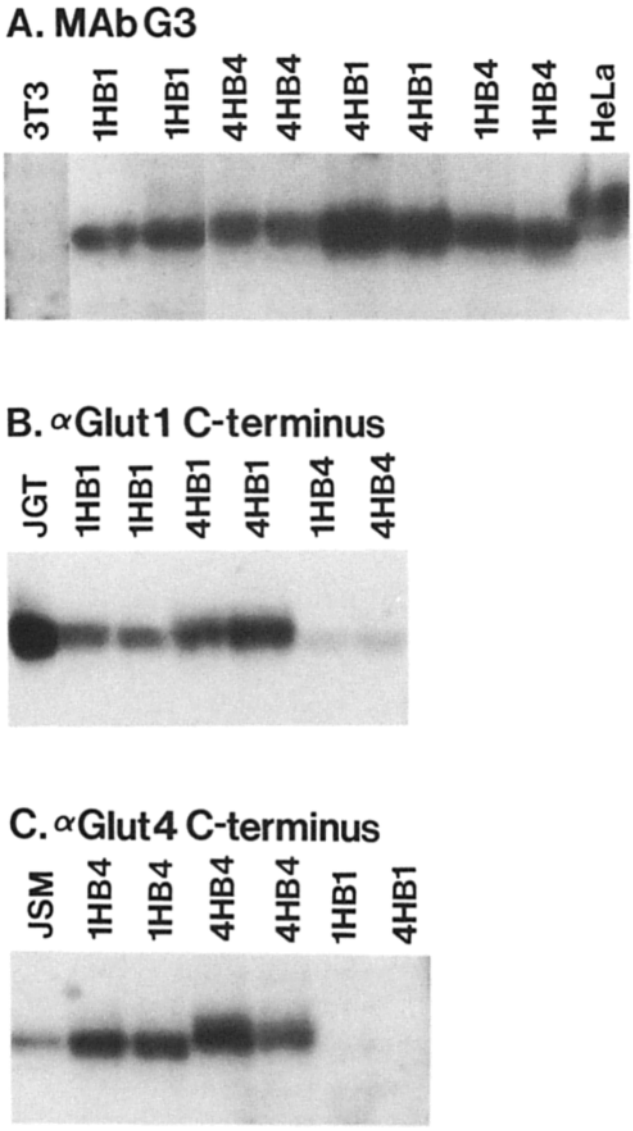

Figure 5. Western blot of clones of NIH 3T3 cells expressing the "tagged" chimeras. Total membranes (50 μg) prepared from NIH 3T3 cells, HeLa cells, NIH 3T3 cells expressing GLUT1 (*JGT*), NIH 3T3 cells expressing GLUT4 (*JSM*), and from clones of NIH 3T3 cells infected with retrovirus containing the cDNAs of the "tagged" chimeras were subject to SDS-PAGE and Western blotting with mAbG3 (*A*), an antibody against the GLUT1 carboxy terminus (*B*), or an antibody to the GLUT4 carboxy terminus (*C*).

lar membranes with mAbG3. Two clones expressing each construct were selected based on the expression of approximately the same amount (within twofold variation) of recombinant transporter. All four "tagged" chimeras, as well as the HeLa cell glucose transporter, were detected by Western blot with mAbG3, whereas the endogenous NIH 3T3 transporter was not (Fig. 5 *A*). As expected, 1HB1 and 4HB1 but not 4HB4 and 1HB4 were recognized by the α-GLUT1 carboxy-terminal antibody (Fig. 5 *B*). This antibody also recognized the endogenous transporter present in transfected and untransfected NIH 3T3 cells (Fig. 5, *B*; and data not shown). The α-GLUT4 carboxy-terminal antibody detected chimeras 4HB4 and 1HB4, but not the glucose transporter present in the parental NIH 3T3 cells (data not shown), or in cells transfected with the chimeras 1HB1 and 4HB1 (Fig. 5 *C*).

NIH 3T3 cells expressing the "tagged" chimeras were used to compare the immunofluorescence staining patterns of the anti-carboxy terminal antibodies to the anti-human mAbG3. The chimeras 1HB1 and 4HB1 were labeled with the α-GLUT1 antibody and mAbG3, whereas the chimeras 4HB4 and 1HB4 were labeled with the α-GLUT4 antibody and mAbG3 (Fig. 6). In general, similar patterns were observed with both mono- and polyclonal antisera, and these confirmed the data presented above. 1HB1 displayed the predominant cell surface staining characteristic of GLUT1 (Fig. 6, *A* and *B*), whereas 4HB4 showed a perinuclear localization identical to that of wild-type GLUT4 (Fig. 6, *D* and *E*). The chimera 1HB4, containing only the COOH-terminal 30 amino acids of GLUT4, showed staining in the perinuclear region similar to that of 4HB4, but with the suggestion of some peripheral immunoreactivity not seen in cells expressing either GLUT4 or 4HB4 (Fig. 6, *J* and *K*). Analysis of the chimera 4HB1 showed a phenotype "intermediate" to the wild-type isoforms, with significant staining of both the plasma membrane and the perinuclear region (Fig. 6, *G* and *H*).

One possible explanation for the perinuclear staining was that the chimeric transporters had accumulated in a biosynthetic compartment such as the Golgi complex. To address this issue, cells were exposed to an inhibitor of protein synthesis before localization of the chimeras by immunofluorescence with mAbG3. Treatment with anisomycin for 1 h did not alter the distribution of the transporters (Fig. 6, *C*, *F*, *I*, and *L*). Identical results were obtained after incubation in anisomycin for up to 4 h and when protein synthesis was inhibited by cycloheximide (data not shown).

As an alternative method for localizing transporters to the cell surface, we and others have adapted a protocol developed for directly visualizing the cytoplasmic face of the plasma membrane (9, 20, 23, 24). Cells grown on poly-D-lysine–coated coverslips were sonified to remove essentially all intracellular contents, leaving "sheets" of PM firmly attached to the cover slips. These PM sheets were fixed with paraformaldehyde and probed with antisera directed against the cytoplasmic epitope "tag" of the chimeric transporters. Since the PM sheets were difficult to visualize by phase microscopy, they were also stained with FITC-WGA. Labeling of 1HB1 with mAbG3 showed the characteristic GLUT1 staining pattern in whole cells and abundant fluorescence in the PM sheets (Fig. 7, *A–C*). In contrast, mAbG3 identified no transporter in PM sheets from cells expressing 4HB4, in spite of the typical perinuclear staining in whole cells and the presence of sheets on the coverslip as confirmed by FITC-WGA staining (Fig. 7, *D–F*). Cells expressing the chimera 4HB1 revealed labeling of PM sheets by mAbG3, though less than that of 1HB1 (Fig. 7, *G–I*). Since both cell lines expressed similar levels of total cellular chimeric transporter, the less intense fluorescence of the PM sheets derived from 4HB1 indicates a smaller percentage of chimeric transporter residing on the cell surface compared to 1HB1. Staining by mAbG3 of PM sheets prepared from 1HB4 fibroblasts was barely detectable (Fig. 7, *J–L*).

The "tagged" chimeric transporters were also introduced into PC12 cells and a similar analysis was performed on pools of G418-resistant cells. Indirect immunofluorescence microscopy with mAbG3 showed patterns of staining of intact PC12 cells expressing 1HB1 or 4HB4 identical to that

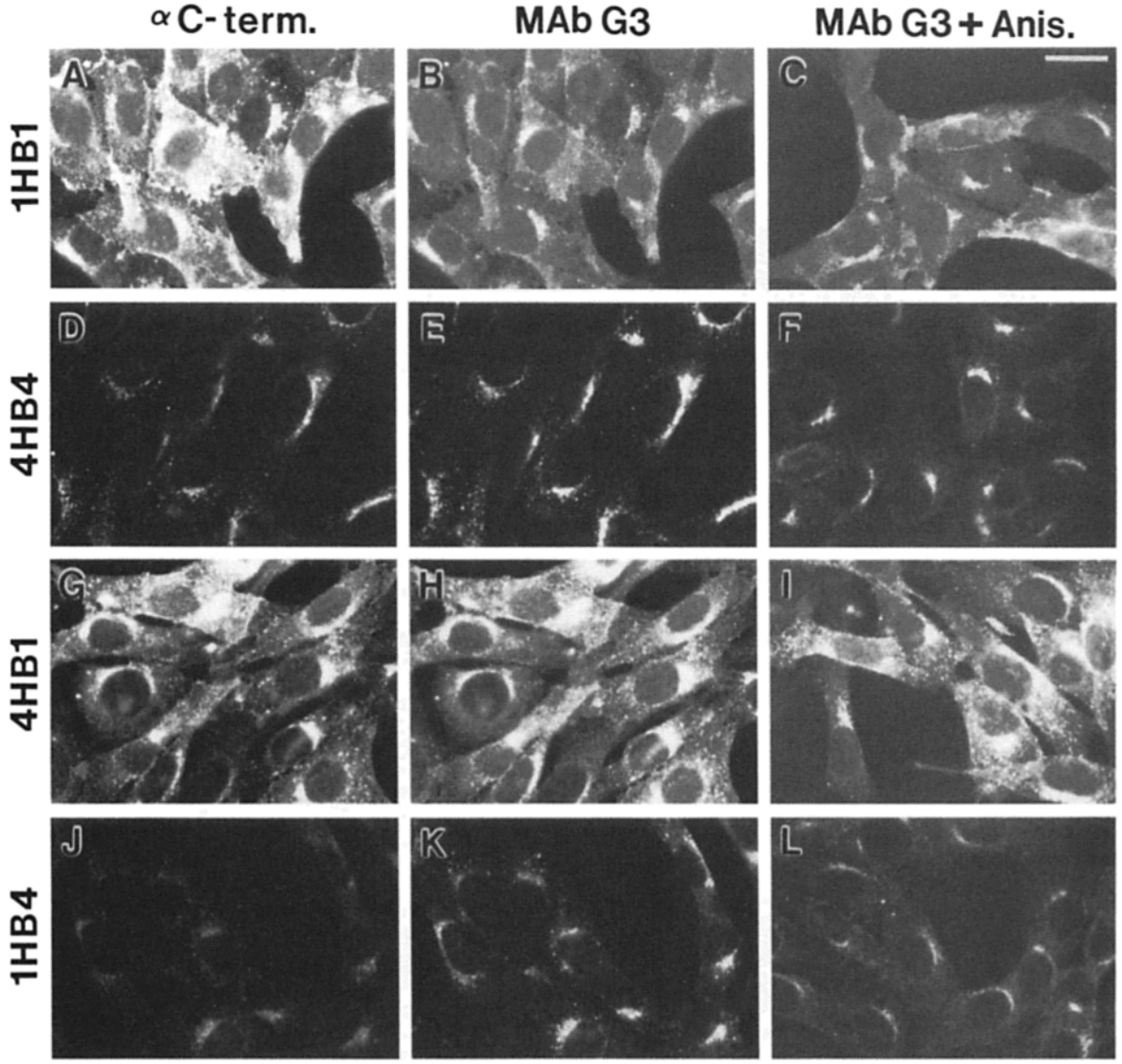

Figure 6. Indirect immunofluorescence of the "tagged" chimeras in NIH 3T3 cells. Clones of NIH 3T3 cells expressing similar levels of the "tagged" chimeric transporters were fixed, permeabilized, and labeled for immunofluorescence. Chimeras 1HB1 (*A* and *B*) and 4HB1 (*G* and *H*) were stained with an antibody to the GLUT1 carboxy terminus and FITC-conjugated goat anti-rabbit antibody (*A* and *G*) and with mAbG3 and rhodamine-conjugated goat anti-mouse antibody (*B* and *H*). Chimeras 4HB4 (*D* and *E*) and 1HB4 (*J* and *K*) were stained with an antibody to the GLUT4 carboxy terminus and FITC-conjugated goat anti-rabbit antibody (*D* and *J*) and with mAbG3 and rhodamine-conjugated goat anti-mouse antibody (*E* and *K*). For *C*, *F*, *I* and *L*, cells were incubated in 100 μM anisomycin for 1 h at 37°C before fixation and staining with mAbG3 and rhodamine-conjugated goat anti-mouse antibody. In parallel experiments, cells infected with vesicular stomatitis virus (VSV) for 3 h and treated with anisomycin displayed negligible staining of VSV G protein in the Golgi complex by immunofluorescence, confirming the effectiveness of the protein synthesis inhibitor (data not shown). For each antibody, the same photographic and printing conditions were used for each chimera. Bar, 20 μM.

of the wild-type GLUT1 or GLUT4, respectively (Fig. 8, *E* and *I*). Plasma membrane sheets prepared from PC12 cells expressing the "tagged" chimeras were also analyzed. Abundant labeling was seen for 1HB1 PM sheets, whereas sheets from cells expressing 4HB4 were not detectable with the same antibody (Fig. 9, *G* and *K*, respectively). 4HB1 was also localized to the cytoplasmic surface of the isolated sheets, but at an abundance less than that for 1HB1 (Fig. 9 *O*), and cells expressing 1HB4 showed slight labeling of PM sheets (Fig. 9 *S*).

These cell lines were also analyzed by scanning laser confocal microscopy; the optical sections shown in Fig. 8 are at approximately mid-nucleus and above the neurites, and thus fluorescence in the latter is generally not visible. Staining of 1HB1 appeared predominantly as a ring of fluorescence at the periphery of the cell (Fig. 8 *F, arrowheads*), whereas labeling of both 4HB4 and 1HB4 was most highly concentrated in the perinuclear region of the cell body (Fig. 8, *J* and *R*, *small arrows*). Note that the distribution of 1HB1 corresponds nicely to the plasma membrane as demarcated by the arrowheads, whereas the central region of the cell is relatively free of transporter. In contrast, 4HB4 and 1HB4 are seen at the tips of the arrows indicative of perinuclear localization, well separated from the arrowheads indicating the

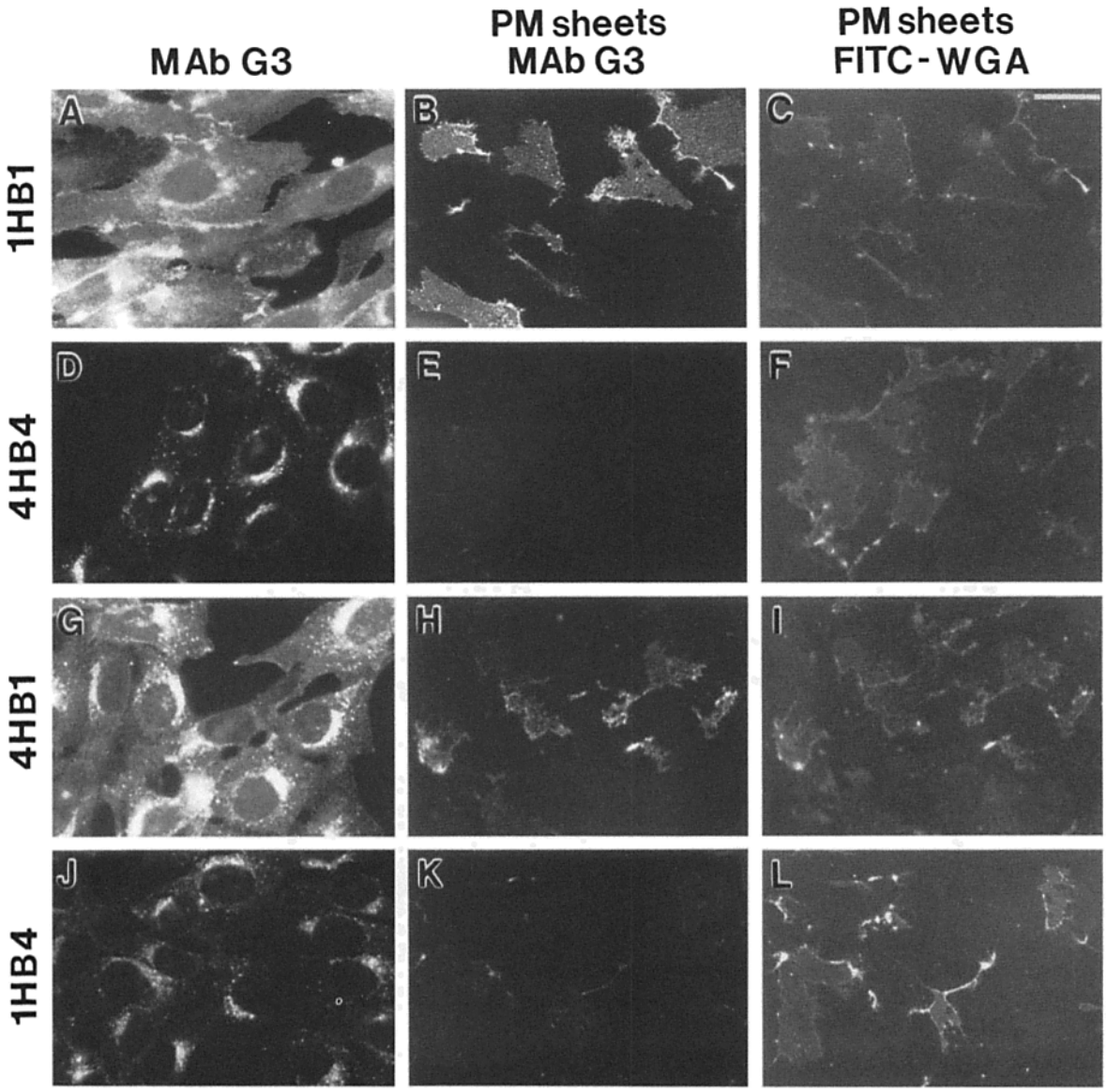

Figure 7. Localization of the "tagged" chimeras in NIH 3T3 cells by labeling of PM sheets. Clones of NIH 3T3 cells stably expressing the "tagged" chimeras were either fixed and stained with mAbG3 for indirect immunofluorescence (*A*, *D*, *G*, and *J*) or were sonified to prepare sheets of PM. These were labeled with mAbG3 and rhodamine-conjugated goat anti-rabbit antibody (*B*, *E*, *H*, and *K*) and with FITC conjugated to WGA (*C*, *F*, *I*, and *L*). For each antibody, the same photographic and printing conditions were used for each chimera. The cells used in this experiment represent different clonal lines than those shown in Fig. 6, but express approximately the same steady state level of chimeric transporter (see Fig. 5). Bar, 20 μM.

cell surface. The chimera 4HB1 again exhibited an intermediate phenotype, showing staining at the cell periphery as well as in the perinuclear region of the cells (Fig. 8 *N*).

Discussion

Although quite similar in primary sequence and predicted secondary structure, the glucose transporter isoforms GLUT1 and GLUT4 are targeted to distinct subcellular locations (4). The unique intracellular sequestration of GLUT4, critical for insulin-stimulated glucose uptake in muscle and adipose tissue, is recapitulated in numerous cell types into which the transporter has been introduced by DNA-mediated gene transfer (12, 17, 23, 26). This provides strong evidence that intrinsic differences between GLUT1 and GLUT4 are recognized by the trafficking machinery of the nondifferentiated cell. As an initial step towards identifying amino acid sequences which dictate isoform-specific targeting, we have studied the subcellular distribution of chimeric GLUT1/GLUT4 transporters stably expressed in two unrelated cell lines. Our results strongly suggest that at least two discontinuous domains disproportionately contribute to the differential sorting of these isoforms.

At the onset of these studies, it seemed likely that substantial care was required to avoid several obvious, potential artifacts. In spite of the remarkable sequence similarity between GLUT1 and GLUT4 (65% amino acid identity), the formal possibility remained that a chimeric transporter could assume a configuration which conferred novel targeting, resulting in a distribution different from either of the parental

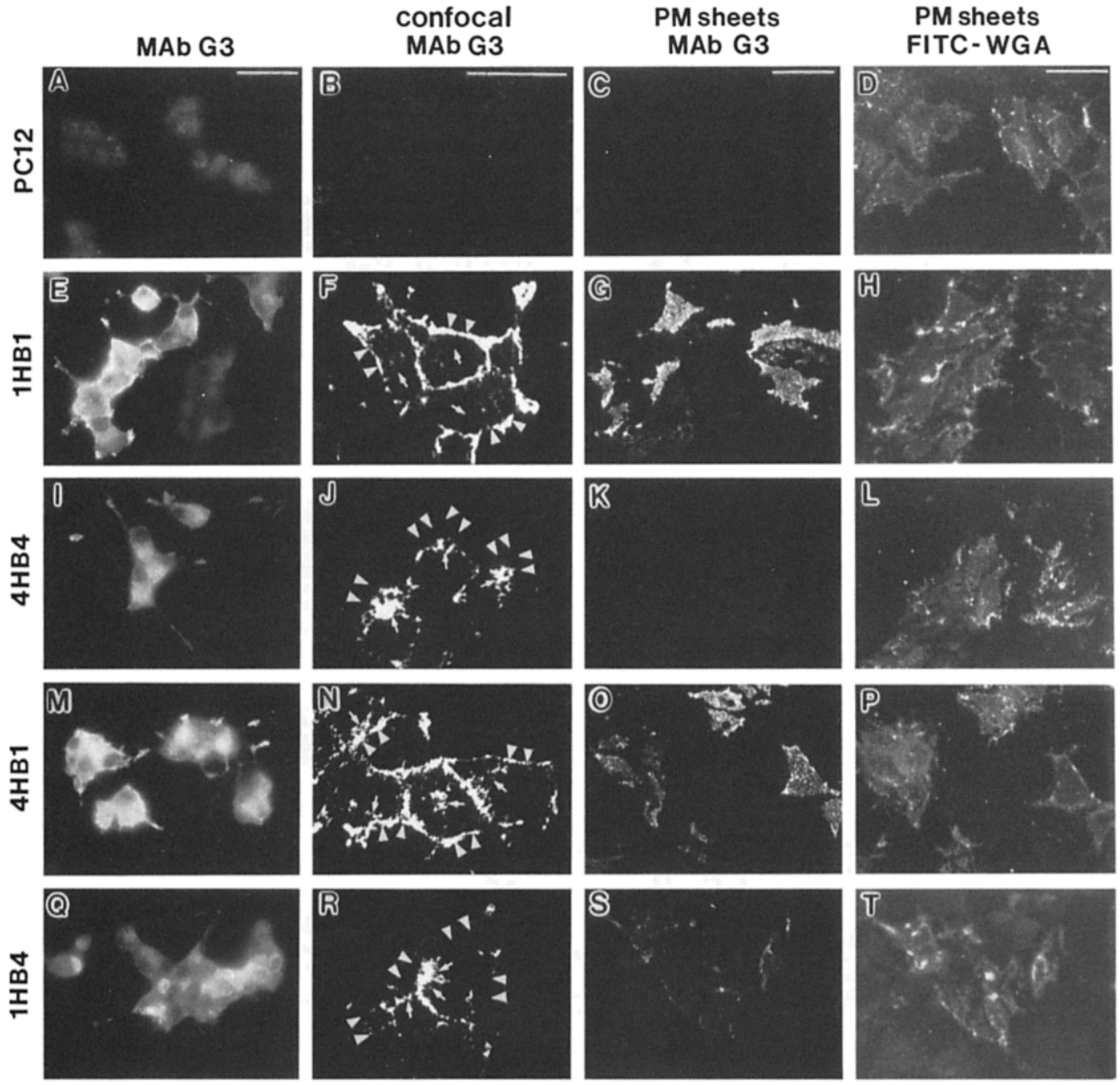

Figure 8. Indirect immunofluorescence and labeling of PM sheets of "tagged" chimeras expressed in PC12 cells. Parental PC12 cells and pools of PC12 cells stably infected with retrovirus containing the cDNAs of the "tagged" chimeras were treated with NGF, fixed, permeabilized, and stained with mAbG3 and rhodamine-conjugated goat anti-mouse antibody, and were analyzed by indirect immunofluorescence (*A*, *E*, *I*, *M*, and *Q*) and by laser scanning confocal microscopy (*B*, *F*, *J*, *N*, and *R*). The periphery of the cells was visualized by labeling of the cell surface with FITC-WGA and is indicated in the figure by arrowheads. Phase micrographs were used to determine the location of the margins of the nuclei, which are indicated by small arrows overlying the nuclei and pointing outward. The same cells were sonified to prepare sheets of PM, which were fixed and labeled with mAbG3 and rhodamine--conjugated goat anti-mouse antibody (*C*, *G*, *K*, *O*, and *S*) and with FITC conjugated to WGA (*D*, *H*, *L*, *P*, and *T*). For each antibody and assay, the same photographic and printing conditions were used for all chimeras. Bars, 20 μM.

wild-type transporters. Several precautions were taken to minimize this likelihood. First, in all cases reciprocal domains were exchanged between isoforms and the resultant chimeras were compared in similar cellular contexts. Second, the mutagenesis strategy employed was such that in no instance was an amino acid residue introduced into a chimera that was not present in a wild-type transporter. Lastly, the chimeric transporters were expressed in two divergent cell types: an undifferentiated fibroblast line and a neuroendocrine cell line with a well-developed regulated secretory pathway. Although NIH 3T3 cells clearly direct GLUT1 and GLUT4 to different subcellular locations, it was possible that the "intermediate" phenotype observed for some of the chimeras might have been related to their expression in an undifferentiated, fibroblastic cell type. Recently, Hudson et al. (18) have found that the rat neuroendocrine cell line PC12 targets GLUT4 but not GLUT1 to a regulated secretory compartment, the large dense core granules, as well as to endosomes. Thus, we also evaluated the distributions of the chimeric transporters in PC12 cells.

Despite these considerations, the initial set of experiments (Figs. 2 and 3) presented several additional problems. Most troublesome was the observation that an apparently dominant signal for subcellular sorting, the carboxy-terminal 30 amino acids, overlapped the epitope recognized by antisera raised against each isoform. Thus, as targeting could not be separated from antibody reactivity, it was impossible to critically confirm the role of this domain using a single antiserum. This was particularly worrisome since the carboxy terminus of the transporter has been implicated as being subject to "epitope masking", i.e., conformational changes which affect its immunoreactivity and consequent apparent subcellular distribution when assayed by microscopy (29). In addition, it was clear that the transporters, both wild-type and chimeric, tended to be expressed at vastly different levels, and it has been suggested that this may influence the resultant staining pattern (2, 17). It was therefore deemed necessary to compare cell lines expressing relatively similar steady-state levels of chimeric transporters, a parameter difficult to ascertain with multiple antibodies and, at least for GLUT1, in the presence of cross-reacting endogenous transporter.

The solution to these problems was to "tag" the recombinant proteins with a nondisruptive neutral epitope, permitting the relatively easy selection of clonal cell lines expressing similar levels of chimeric transporters, as well as allowing a direct comparison of the staining patterns of reciprocal chimeras. To accomplish this without introducing foreign sequences into the recombinant transporters, we used a human-specific mAb which recognizes a region of GLUT1 in which there is only one, conservative amino acid difference between the rat and human transporters. We confirmed that changing Arg 239 to His in the rat GLUT1 sequence was sufficient to confer reactivity with mAbG3, and that this antisera did not detect the transporter endogenous to NIH 3T3 or PC12 cells (Figs. 4, 5, and 8). The initial experiments suggested that the site at which the human-specific GLUT1 epitope was located appeared not to affect targeting (Figs. 2 and 3). This was confirmed by demonstrating that the subcellular distribution of 4HB4 was indistinguishable from that of wild-type GLUT4 in numerous cell types (Figs. 7–9, and data not shown). Thus, the construction of the "human-tagged" set of chimeric transporters (Fig. 1, Group III) served to eliminate potential artifacts due to significant variability in levels of expression and to differences in immunoreactivity at the carboxy terminus. In addition, the similarity in staining patterns with antisera directed against different regions of the chimeric transporters reduces the likelihood of "epitope-masking" as a confounding factor (Fig. 6).

The last cause for concern addressed by experiments using the "tagged" chimeras was the lack of resolution and consequent ambiguity of light microscopy (Figs. 2 and 3). Since mAbG3 was not an adequate reagent for immunoEM (Hudson, A. W., K. J. Verhey, and M. J. Birnbaum, unpublished observations), we chose to use labeling of PM sheets as an unambiguous method of assessing cell surface abundance of the transporters. This assay led to dramatic differences in the immunofluorescence staining of cells expressing equivalent amounts of 1HB1 and 4HB4 (Figs. 8 and 9).

The clearest localization of the chimeras occurred when both the amino-terminal 183 amino acids and the carboxyl-terminal 30 amino acids were contributed by the same isoform (chimeras 1411, 4144, 1A41, 4A14, 1HB1, and 4HB4). Chimeras which contained the amino-terminal 183 amino acids of GLUT1 and the last 30 amino acids of GLUT4 (1444, 1144, 1114, 1A14, and 1HB4) were directed primarily to the perinuclear region, similar to GLUT4. The reciprocal chimeric transporters, in which the amino-terminal 183 amino acids were contributed by GLUT4 and the carboxyl-terminal 30 amino acids by GLUT1 (4441, 4411, 4111, 4A41, and 4HB1), consistently showed an intermediate phenotype, localizing to both the perinuclear region and the cell surface. Taken together, these results indicate that the cytoplasmic carboxy terminus contains the dominant determinant of subcellular localization for transporters expressed in NIH 3T3 or PC12 cells, but targeting information also resides in the amino third of the protein.

Two laboratories have recently reported studies aimed at identifying sorting signals that distinguish GLUT4 from GLUT1. Piper et al. (23) expressed recombinant transporters in hamster CHO fibroblasts using a Sindbis virus transient expression system. Antibodies directed against the carboxy terminus of each chimera were used to localize them by confocal immunofluorescence, EM, and the PM sheet assay. The authors concluded that the amino-terminal 41 amino acids of GLUT4 are necessary and sufficient for intercellular sequestration of this transporter. Asano et al. (2) replaced the amino terminus of GLUT4 with increasing amounts of GLUT1, maintaining a GLUT4 carboxy terminus. Chimeric proteins stably expressed in CHO cells were localized by immunofluorescence and EM using an antibody against GLUT4. Two domains were determined to be important for the intracellular sequestration of GLUT4: one containing part of MS2 and all of MS3, and a second consisting of the carboxy-terminal half of the extracellular loop between MS7 and MS8 and all of MS8. We are unable to explain with certainty the discrepancies between the two previous studies, or between those studies and the data reported herein. In contrast to previously published studies, we have determined that the carboxy-terminal 30 amino acids of the transporter also contain information dictating subcellular distribution. Replacement of this region of GLUT1 by GLUT4 diverted the predominant fraction of the former from the plasma membrane to the perinuclear region. Several methodological differences may account for the discrepancies among these three studies, including the type of cell in which the chimeras are expressed and the amount of heterologous transporter. Perhaps the most critical difference between this and previous studies is that in the latter the majority of relevant chimeras were localized exclusively with an antibody to the carboxy terminus of GLUT4, and thus did not systematically explore the contribution of this region to targeting. In experiments using a single antibody to an epitope located in the large cytoplasmic loop of GLUT1, a region all investigators agree is not critical for isoform-specific targeting, we were able to directly compare chimeras in which the only difference was the carboxy-terminal 30 amino acids. During the preparation of this manuscript, we became aware of two preliminary, independent reports which appear to agree with our findings: the carboxy terminus of GLUT4 was found to contain information capable of sequestering chimeric transporters to an intracellular compartment in CHO cells or *Xenopus laevis* oocytes (7; Marshall, B., M. Strube, and M. Mueckler. 1993. *Diabetes.* 42(Suppl.). 1:13a).

The identification of two domains of the transporter as important for targeting suggests several experimentally testable models. One possibility is that these are redundant signals, with both cooperating to ensure efficient targeting of the transporters. Alternatively, these two domains may function independently at distinct sorting steps carried out in different regions of the cell. Lastly, these two regions may be juxtaposed in the native transporter to form a single, discontinuous sorting signal. In addition to distinguishing these possibilities, future experiments will be directed at further resolving the critical sequences in each region, as well as determining their relevance to insulin-stimulated transporter translocation.

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