Insulin-sensitive Targeting of the GLUT4 Glucose Transporter in L6 Myoblasts Is Conferred by its COOH-terminal Cytoplasmic Tail

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Abstract. The GLUT4 glucose transporter appears to be targeted to a unique insulin-sensitive intracellular membrane compartment in fat and muscle cells. Insulin stimulates glucose transport in these cell types by mediating the partial redistribution of GLUT4 from this intracellular compartment to the plasma membrane. The structural basis for the unique targeting behavior of GLUT4 was investigated in the insulin-sensitive L6 myoblast cell line. Analysis of immunogold-labeled cells of independent clonal lines by electron microscopy indicated that 51–53% of GLUT1 was present in the plasma membrane in the basal state. Insulin did not significantly affect this distribution. In contrast, only 4.2–6.1% of GLUT4 was present in the plasma membrane of basal L6 cells and insulin increased this percentage by 3.7–6.1-fold. Under basal conditions and after insulin treatment, GLUT4 was detected in tubulovesicular structures, often clustered near Golgi stacks, and in endosome-like vesicles. Analysis of 25 chimeric transporters consisting of reciprocal domains of GLUT1 and GLUT4 by confocal immunofluorescence microscopy indicated that only the final 25 amino acids of the COOH-terminal cytoplasmic tail of GLUT4 were both necessary and sufficient for the targeting pattern observed for GLUT4. A dileucine motif present in the COOH-terminal tail of GLUT4 was found to be necessary, but not sufficient, for intracellular targeting. Contrary to previous studies, the NH_2 terminus of GLUT4 did not affect the subcellular distribution of chimeras. Analysis of a chimera containing the COOH-terminal tail of GLUT4 by immunogold electron microscopy indicated that its subcellular distribution in basal cells was very similar to that of wild-type GLUT4 and that its content in the plasma membrane increased 6.8–10.5-fold in the presence of insulin. Furthermore, only the chimera containing the COOH terminus of GLUT4 enhanced insulin responsive 2-deoxyglucose uptake. GLUT1 and two other chimeras lacking the COOH terminus of GLUT4 were studied by immunogold electron microscopy and did not demonstrate insulin-mediated changes in subcellular distribution. The NH_2-terminal cytoplasmic tail of GLUT4 did not confer intracellular sequestration and did not cause altered subcellular distribution in the presence of insulin. Intracellular targeting of one chimera to non-insulin-sensitive compartments was also observed. We conclude that the COOH terminus of GLUT4 is both necessary and sufficient to confer insulin-sensitive subcellular targeting of chimeric glucose transporters in L6 myoblasts.

GLUCOSE transport into cells is facilitated by the family of glucose transporter isoforms (Bell et al., 1990; Mueckler, 1994; Gould and Holman, 1993). GLUT4, expressed only in fat and muscle, is responsible for the increase in glucose uptake in these tissues in the presence of insulin. This is caused by its translocation from an intracellular compartment to the plasma membrane in response to insulin. In contrast to GLUT1, which is present in the plasma membrane as well as in intracellular compartments and therefore contributes to basal glucose uptake, GLUT4 is virtually excluded from the plasma membrane in the absence of insulin. Insulin causes a small enrichment in the GLUT1 content of the adipocyte plasma membrane (Piper et al., 1991), on the order of that observed in response to insulin or insulin-like growth factors, for several membrane proteins, such as the α-2 and β-1 subunits of the Na^+, K^+-ATPase (Hundal et al., 1992), the IGF-II/mannose-6-phosphate receptor (Damke et al., 1992), and the transferrin receptor (Damke et al., 1992). However, insulin does not change the distribution of either GLUT1 or Na^+-K^+-ATPase α-1 subunits in plasma membranes of red or white muscles (Marette et al., 1992*b*). In contrast, in response to insulin, GLUT4 is enriched in the plasma membrane, and glucose

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transport activity is augmented, two- to eightfold in muscle (Smith and Lawrence, 1984; Marette et al., 1992*a*,*b*; Wilson and Cushman, 1994) and up to 30-fold in fat (Holman et al., 1990). The distinctive subcellular distributions of GLUT1 and GLUT4 are reflected in the differential effects of overexpression of these transporters on glucose transport activity. Overexpression of GLUT1 in a variety of systems, including 3T3-L1 fibroblasts (Haney et al., 1991), CHO cells (Asano et al., 1992), *Xenopus* oocytes (Marshall et al., 1993*a*), L6 myoblasts (Robinson et al., 1993), and transgenic mouse muscle (Marshall et al., 1993*b*), increases basal glucose uptake but does not enhance insulin responsiveness. In contrast, overexpression of GLUT4 in insulin-sensitive L6 myoblasts increases insulin-responsive glucose uptake without significantly affecting basal uptake (Robinson et al., 1993). The two transporters exhibit differential targeting despite the level of identity (65%) and similarity (76%) in amino acid sequence they share. We set out to identify the structural basis for the unique intracellular targeting of GLUT4 in insulin-sensitive cells by studying the expression of chimeric glucose transporters composed of complementary regions of GLUT1 and GLUT4.

Several earlier studies indicated that GLUT4 targeting is isoform-specific and independent of cell type (Haney et al., 1991; Hudson et al., 1992; Shibaski et al., 1992; Kotliar and Pilch, 1992). This observation led to the use of insulin-insensitive cells to delineate domains of GLUT4 that cause intracellular sequestration. Five different groups have reported three different and mutually contradictory sets of results derived from targeting of chimeric glucose transporters. Piper et al. (1992) used Sindbis virus to overexpress chimeras in CHO cells and concluded that the NH_2-terminus of GLUT4 confers intracellular sequestration. Asano et al. (1992) used stable transfection of CHO cells and concluded that regions in the first and third quarters of GLUT4, but neither its NH_2 terminus nor its COOH terminus, contain intracellular targeting signals. Marshall et al. (1993*a*) demonstrated that in *Xenopus* oocytes, the COOH terminus conferred sequestration of fully glycosylated chimeras in a low-density intracellular membrane compartment. The region between amino acids 24 and 132 of GLUT4 contributed to sequestration, but this was attributed at least in part to an altered rate of processing. Czech et al. (1993) demonstrated by transient transfection of COS-7 cells and stable transfection of CHO cells, and Verhey et al. (1993) demonstrated by stable transfection of NIH 3T3 and PC12 cells, that the COOH terminus of GLUT4 confers intracellular targeting. These studies also indicated a less important signal between amino acids 53 and 199 (Czech et al., 1993) or between amino acids 1 and 183 (Verhey et al., 1993) of GLUT4. Verhey and Birnbaum (1994) recently identified a dileucine motif of the COOH terminus as necessary for intracellular targeting of GLUT4. Corvera et al. (1994) have recently presented evidence that the dileucine motif operates as an endocytic signal. Thus, a consensus is emerging that the COOH terminus of GLUT4 is important for intracellular targeting. However, since these studies were carried out in cells that do not respond to insulin, they all include the assumption that intracellular accumulation of a chimeric transporter in insulin-insensitive cells accurately reflects targeting to the native insulin-sensitive intracellular compartment of a fat or muscle cell. A recent study suggests the tenuous nature of this assumption by showing that chimeras comprised of domains of dipeptidyl peptidase IV and aminopeptidase N, both of which normally target to the plasma membrane, appeared to target to the *trans*-Golgi network (Low et al., 1994). This aberrant result was attributed to inefficient transport from the Golgi to the cell surface.

We considered it important for our studies of specific targeting domains of chimeric glucose transporters to determine the subcellular distribution at steady state in insulin-sensitive cells that are expressing little or no GLUT4 and are expressing exogenous glucose transporters at approximately the level at which glucose transporters are naturally abundant. These considerations take into account phenomena that might cause misleading results, such as dimerization of chimeras with endogenous GLUT4 and saturation of specific sorting systems. The study reported here was thus carried out in L6 myoblasts. Upon spontaneous differentiation into myotubes, these cells express GLUT4 and become insulin sensitive; however, maintained as myoblasts in 10% serum, differentiation is inhibited and very little GLUT4 is expressed (Mitsumoto et al., 1992). Lawrence et al. (1992) showed that expression of exogenous GLUT4 at the myoblast stage confers insulin responsiveness, and subsequently confirmed this in three additional clonal lines of L6 cells overexpressing GLUT4 (Robinson et al., 1993). Thus, these cells appear to contain all of the machinery necessary for insulin-sensitive trafficking of GLUT4, and to exhibit differential targeting and insulin-responsiveness of transfected GLUT1 and GLUT4 (Robinson et al., 1993). We therefore reasoned that L6 myoblasts might represent a suitable system to define the region(s) of GLUT4 important in targeting to an insulin-sensitive intracellular compartment. In doing so, we show that targeting of chimeric glucose transporters to non–insulin-sensitive intracellular compartments can occur. Our results show that insulin-sensitive targeting of GLUT4 is conferred by its COOH-terminal cytoplasmic tail. We also present evidence that the dileucine motif within the COOH-terminal cytoplasmic tail is necessary, but not sufficient, for insulin-sensitive targeting. We find no role for the NH_2-terminal cytoplasmic tail of GLUT4.

Materials and Methods

Cell Culture and 2-Deoxyglucose Uptake Measurements

L6 myoblasts (American Type Culture Collection, Rockville, MD) were grown in alpha-minimal essential medium supplemented with 10% FCS (Robinson et al., 1993), 50 U/ml penicillin, 50 μg/ml streptomycin, and 250 ng/ml amphotericin B in an atmosphere of 5% CO_2 at 37°C. All experiments were performed on the day before confluence would have been attained, before any sign of differentiation. Medium was changed one day before experiments. [^{3}H]-2-Deoxyglucose uptake was measured as previously described (Tordjman et al., 1989). Non–carrier-mediated uptake was measured in the presence of 20 μM cytochalasin B. Cells were incubated in serum-free medium for 2 h before measurement. Insulin-stimulated uptake was measured after 20-min incubation with 10^{-6} M insulin (Robinson et al., 1993) in serum-free medium. Protein content was measured by the bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL).

Construction of Glucose Transporter Expression Vectors

Chimeric glucose transporter cDNAs were prepared using recombinant PCR (Vallette et al., 1989). The amino acids were numbered according to previously published sequences (Mueckler et al., 1985; Birnbaum, 1989;

Charron et al., 1989; Fukumoto et al., 1989; James et al., 1989; Kaestner et al., 1989). Chimeras consisted partially of human GLUT1 and partially of rat GLUT4 and were constructed by engineering junctions at analogous points in the two transporters. The nomenclature for chimeras denotes the composition of the chimera beginning at the NH_2 terminus. Thus, a "14" chimera begins with GLUT1 and ends with GLUT4; a "141" chimera begins and ends with GLUT1 but contains GLUT4 within it. The number(s) following the initial designation indicate the amino acid number, according to the transporter contributing the NH_2 terminus, of the junction(s). Thus, the chimera 14-450 contains the first 450 amino acids of GLUT1, and the remainder is GLUT4. The converse chimera, at the same junction, was denoted as 41-466 because it contains the first 466 amino acids of GLUT4, but the rest is GLUT1. Three-part chimeras are also numbered according to the transporter contributing the NH_2 terminus, so that, for example, the chimera 141-450,464 contains the first 450 amino acids of GLUT1, then the corresponding next 13 amino acids of GLUT4, then resumes with amino acid 464 of GLUT1. Fig. 1 illustrates the twelve junctions used in construction of the chimeras. Junction H was used for chimeras 14-450 and 41-466, while junctions H and I were used for chimera 141-450, 464. The first round of PCR was carried out using GLUT1 or GLUT4 cDNA as appropriate, as template. In most cases, GLUT4 cDNA had been previously altered by recombinant PCR to introduce, in a reciprocal fashion, the analogous region of the intracellular loop amino acids 233-246 of human GLUT1, which contains the epitope for the species-specific monoclonal antibody 37.6. One oligonucleotide primer consisted of 15 nucleotides on each side of the chosen junction; the second oligonucleotide was the appropriate 5' or 3' primer conveniently designed to include restriction enzyme sites to facilitate subcloning. Full-length chimeric cDNAs produced by the recombinant PCR reaction were then subcloned into the mammalian expression vector Rc/CMV (Invitrogen, San Diego, CA), which contains the neomycin resistance gene. Several constructs, including 14-450, were completely sequenced to confirm the fidelity of PCR. Site-directed mutagenesis was carried out according to established protocols (Clontech, Palo Alto, CA). The mutations introduced into the chimera 14-450 to create the chimera 14-450 (L489·, L490S) are in the dileucine motif located at amino acids 489 and 490 of GLUT4.

Transfection of Mammalian Cell Lines

Confluent L6 myoblasts were split 1:5 into 10-cm dishes 24 h before transfection with a calcium phosphate precipitate containing 25 μg of recombinant pRc/CMV, as described previously (Haney et al., 1991). Cells were exposed to 0.8 mg/ml G418 (active fraction 0.51 mg/ml) (GIBCO BRL, Gaithersburg, MD) during selection and were subsequently maintained in medium containing 0.4 mg/ml G418 to limit growth of revertants. Constructs intended for 2-deoxyglucose uptake experiments and electron microscopy were used to generate clonal cell lines. Approximately 100 individual G418-resistant colonies were isolated with cloning rings after 2 wk of selection, grown to confluence in 24-well tissue culture dishes, screened by Western blotting, and selected colonies were then grown in mass. All selected clonal cell lines expressed exogenous transporters at levels less than or approximately the same as the levels of endogenous glucose transporters.

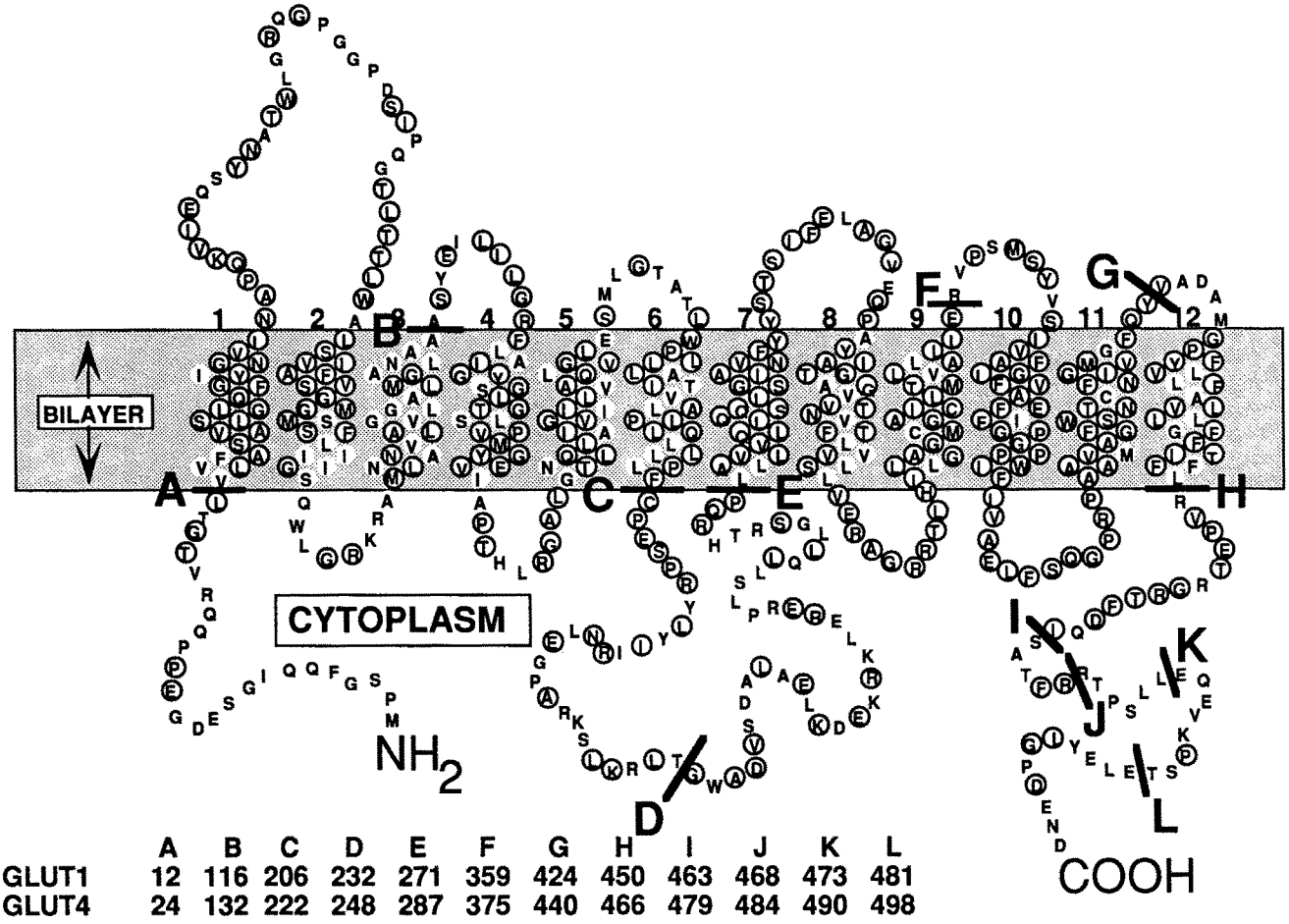

Figure 1. Junctions used in the construction of GLUT1-GLUT4 chimeras. The postulated topology and amino acid sequence of GLUT4 are shown (James et al., 1989). Amino acid residues that are identical in GLUT1 and GLUT4 are circled. The two transporters are 65% identical in sequence. The twelve junctions used in the construction of the 25 chimeric glucose transporters reported here are labeled (*A-L*). Also shown for GLUT1 and GLUT4 is the number of amino acids in each transporter from the NH_2 terminus to each junction. For example, GLUT1 has 450 amino acids NH_2-terminal to junction H, while GLUT4 has 466 amino acids NH_2-terminal to junction H. Therefore, the converse chimeras at this junction are named 14-450 and 41-466 (see text).

No cell lines generated using this vector expressed exogenous transporter at levels higher than the range of levels of endogenous transporters. Most constructs intended for analysis only by confocal microscopy were studied as pooled permanent transfectants; rather than isolating individual clones, G418-resistant cells were trypsinized after 10 d of selection and grown on coverslips for study. Only a small fraction of pooled G418-resistant cells express exogenous glucose transporter.

Western Blot Analysis and Confocal Immunofluorescence Microscopy

Transfected cell lines were characterized by SDS-PAGE and Western blot analysis, using rabbit polyclonal antisera to human GLUT1 (F350), mouse GLUT3 (mGLUT3), and rat GLUT4 (F349), as described previously (Haney et al., 1991). In each case, the antigens for these sera were synthetic peptides corresponding to the final 16 amino acids of the COOH terminus, and each antibody is highly specific. A species-specific monoclonal antibody to human GLUT1, 37.6, directed against an epitope including his-239 of human GLUT1, within its intracellular loop, was a kind gift of Dr. G. Lienhard (Dartmouth Medical School), (Allard and Lienhard, 1985). Except for GLUT4 and the constructs 14-232 and 141-116, 272, all constructs contained the monoclonal epitope.

The quantities of the glucose transporter proteins expressed in transfected cell lines were determined by Western blot, with comparison to standards consisting of purified human erythrocyte glucose transporter to quantitate GLUT1 or cytochalasin B-titered low density microsomes from rat adipocytes to quantitate GLUT4, as described previously (Haney et al., 1991). For GLUT3, rat brain was used as a standard, and results are presented as GLUT3 content relative to untransfected L6 myoblasts. Signals were quantitated by densitometry.

Confocal immunofluorescence microscopy was carried out as previously described (Haney et al., 1991), using COOH-terminal peptide immunoaffinity purified antibodies F350 and F349 (Parekh et al., 1989) against GLUT1 and GLUT4, respectively. Protein A-purified monoclonal antibody 37.6, directed against the human GLUT1 epitope contained in nearly all chimeras, was used for specific detection of exogenous transporters.

Electron Microscopy

Confluent 15-cm dishes of L6 myoblasts were rinsed three times with PBS and treated with 0.05% trypsin/0.2% EDTA. Trypsinized cells were centrifuged at 300 *g* for 5 min at 4°C and fixed with 2% paraformaldehyde/0.2% glutaraldehyde in PBS, pH 7.2, at room temperature for 2 h. This brief trypsin exposure did not affect the subcellular distribution of GLUT1 or GLUT4 (data not shown). Cells were embedded in 10% gelatin as described by Slot and Geuze (1988) but without fixation of the gelatin. After pelleting the cells, the gelatin was solidified on ice. Blocks for ultracryotomy were prepared and infused with 2.3 M sucrose for 2 d. Ultrathin sections were prepared and immunolabeled as described by Slot et al. (1991*a*) with the following modifications. 10% fetal bovine serum was used in the blocking buffer in place of 1% BSA. Immunolabeling with affinity-purified antibodies was carried out for 2 h. Incubation with secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) 18 nm goat anti-rabbit IgG/Au for the rabbit polyclonal antibodies F349 and F350, and 12 nm goat anti-mouse IgG/Au for the mouse monoclonal antibody 37.6, was carried out for 1 h. Sections were stained with uranyl acetate and embedded in methyl cellulose according to a modification of the Tokuyasu method (Tokuyasu, 1980) introduced by Griffiths et al. (1984). Specimens were viewed and photographed using a Zeiss 902 electron microscope. Intact cells with adequate morphology were selected for quantitation of immunogold labeling. Particles within 20 nm of the plasma membrane were defined as plasma membrane for purposes of quantitation (Slot and Geuze, 1988; Slot et al., 1991*a*). Particles within clusters were counted individually. For each condition (basal vs. insulin) of each independent cell line, ten cells were counted; these were from two to four grids of a single section from a single fixation. Up to three independent cell lines per construct were studied.

Results

Rat L6 myoblasts were stably transfected with a plasmid encoding both neomycin resistance and one of a series of wild-type and chimeric glucose transporters. Cells resistant to G418 were studied either as pooled permanent transfectants or, if intended for more detailed study, clonal cell lines were isolated. Nearly all chimeric glucose transporters were designed to include the epitope for 37.6, a well characterized species-specific monoclonal antibody against human GLUT1 (Allard and Lienhard, 1985; Haney et al., 1991; Verhey et al., 1993). Verhey et al. (1993) used this strategy to permit the use of a single antibody to characterize chimeric glucose transporter targeting. This antibody, which was used in screening colonies by Western blotting, recognizes only exogenous transporters. An additional advantage of using this antibody, which recognizes an epitope in the intracellular loop between the sixth and seventh transmembrane domains of GLUT1, is that the COOH-terminal epitope recognized by the polyclonal antibody F349 has been suspected of being "masked" under certain conditions (Smith et al., 1991).

The levels of both endogenous and exogenous glucose transporters varied in clonal cell lines (Fig. 2). The F350 and F349 sera recognizes endogenous GLUT1 and GLUT4, re-

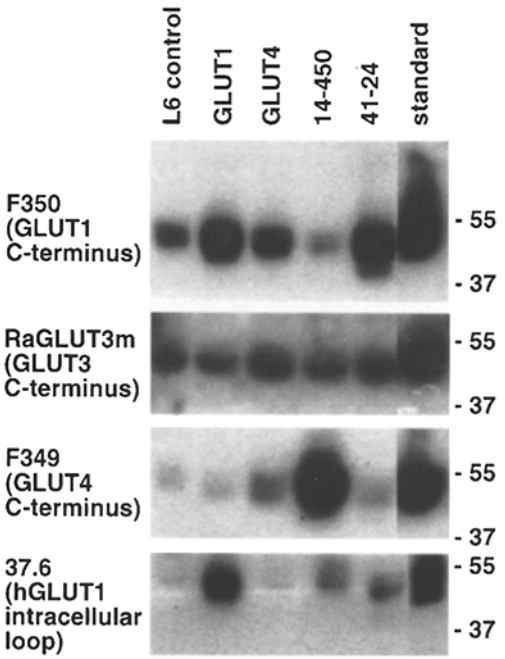

Figure 2. Expression of wild-type and chimeric glucose transporters in L6 myoblast cell lines. Identical 20 μg aliquots of total detergent-soluble protein fractions were electrophoresed on 10% polyacrylamide gels and the gels subjected to Western blotting as described in Materials and Methods. The first blot was incubated with F350, a polyclonal antibody that recognizes both endogenous GLUT1 and chimeras containing the GLUT1 COOH terminus. The second blot was incubated with RaGLUT3m, which recognizes the COOH terminus of GLUT3. The third blot was incubated with F349, which recognizes endogenous GLUT4 and chimeras containing the GLUT4 COOH terminus. The final blot was incubated with 37.6, a species-specific monoclonal antibody that recognizes only exogenous transporters containing the appropriate epitope of the human GLUT1 intracellular loop, which was engineered into nearly all chimeras. Standards were: for F350, 30 ng of purified human erythrocyte glucose transporter; for RaGLUT3m, 50 μg of rat brain detergent-soluble protein fraction; for F349, a cytochalasin B-titered rat LDM fraction containing 5 ng of GLUT4; and for 37.6, 20 ng of purified human erythrocyte glucose transporter.

spectively, as well as exogenous transporters containing the respective COOH-terminal epitopes, while the species-specific monoclonal antibody 37.6 recognizes only exogenous transporters containing histidine-239 of the intracellular loop midway through the human transporter (Davies et al., 1987), between junctions D and E (see Fig. 1). Densitometry revealed that the level of total GLUT1 expression in the transfected cell line was approximately 2.7-fold above the endogenous level, at slightly less than 0.1% of total protein. GLUT4 was expressed at a level eightfold greater than that in untransfected cells, but constituted less than 0.01% of total protein. The highest level of expression of any exogenous transporter was about 0.1% of total protein. The level of endogenous GLUT3 expressed in the various transfected cell lines was variable.

The targeting of GLUT1 and GLUT4 in lines overexpressing these transporters as revealed by laser confocal immunofluorescence microscopy is shown in Fig. 3. For GLUT1, staining is shown both for the polyclonal and for the species-specific monoclonal antibody. As in 3T3-L1 fibroblasts (Haney et al., 1991), GLUT4 was effectively excluded from the plasma membrane. In contrast, there was abundant plasma membrane staining for GLUT1. GLUT4 was present in a discrete perinuclear compartment and was observed in a punctate pattern throughout the cytoplasm. It is evident from the low power views that GLUT1 also displayed intracellular staining, although not always in the focal plane of the laser.

The strategy used to determine the intracellular targeting domains of GLUT4 was to initially use confocal immunofluorescence microscopy to examine the intracellular targeting activity of each half and then each quarter of the protein, and then to focus on areas of interest for further study. Fig. 4 shows the distribution of 41-222 (junction C), 41-248 and 14-232 (junction D), and 14-271 (junction E). Only the COOH-terminal half of GLUT4 conferred intracellular targeting and exclusion from the plasma membrane. The constructs containing the NH_2-terminal half of GLUT4 targeted similarly to wild-type GLUT1. Fig. 4 also shows the distribution of 41-132 (junction A), 141-116,272 (junctions B and E), 141-271,360 (junctions E and F), and 14-359 (junction F). These constructs each contain one of the four quarters of GLUT4 in a GLUT1 backbone. Only the fourth quarter conferred intracellular targeting and exclusion from the plasma membrane. The constructs containing one of the first three quarters targeted similarly to wild-type GLUT1.

Within the fourth quarters of GLUT1 and GLUT4, the final three transmembrane domains are strikingly homologous (Fig. 1), and the major divergence between the two transporters is within the COOH-terminal cytoplasmic

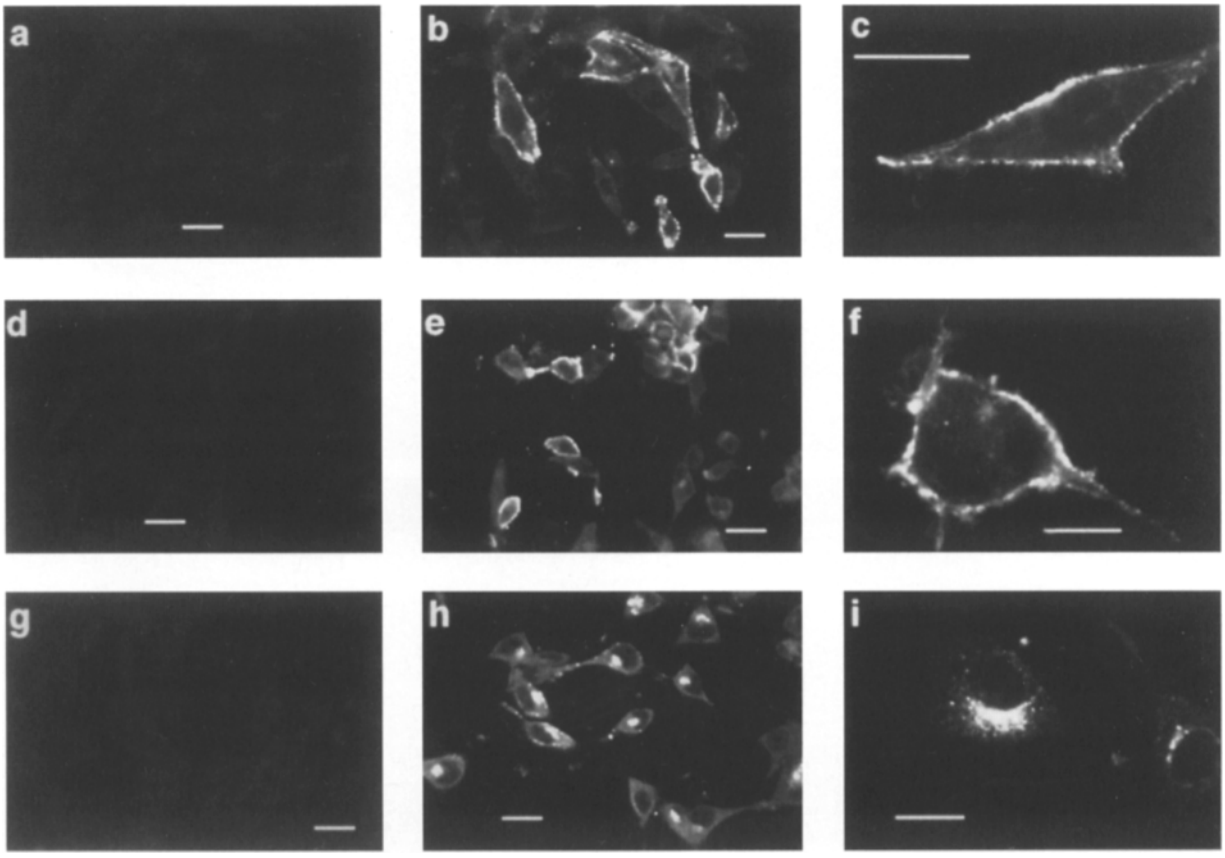

Figure 3. Confocal immunofluorescence microscopy of clonal lines of transfected L6 myoblasts expressing GLUT1 and GLUT4. Representative lower and higher magnification fields of untransfected control cells and of clonal cell lines overexpressing GLUT1 and GLUT4 are shown. For GLUT1, results are shown for both the polyclonal antibody F350, which recognizes the COOH terminus of GLUT1, and the species-specific monoclonal antibody 37.6, which recognizes an epitope of the intracellular loop midway through the transporter. For GLUT4, results are shown for the polyclonal antibody F349, which recognizes the COOH terminus of GLUT4. (*a–c*) Polyclonal antibody F350: (*a*) control, (*b* and *c*) GLUT1; (*d–f*) monoclonal antibody 37.6: (*d*) control, (*e* and *f*) GLUT1; (*g–i*) polyclonal antibody F349: (*g*) control, (*h* and *i*) GLUT4. Bars, 20 μm.

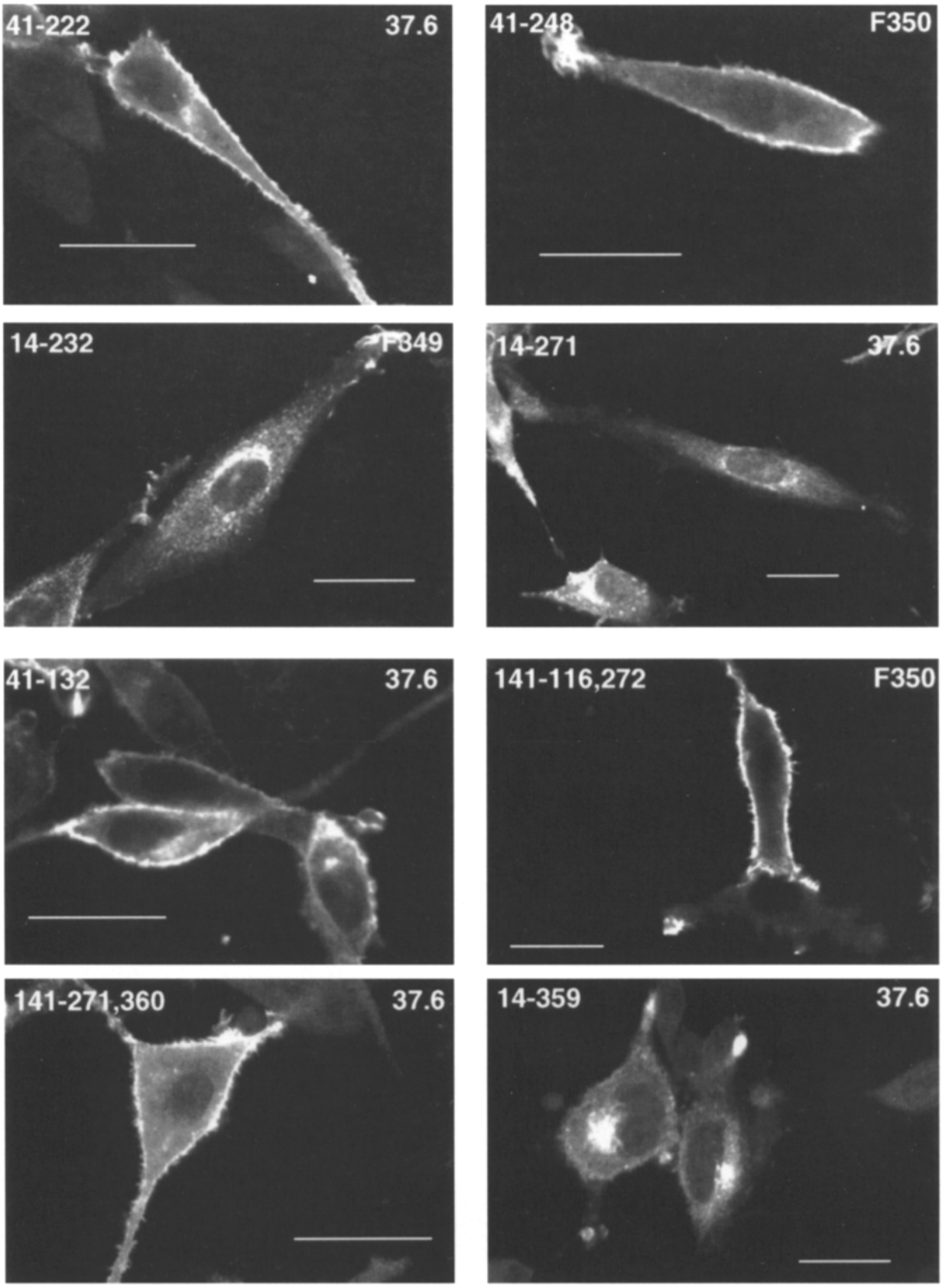

Figure 4. Confocal immunofluorescence microscopy of transfected L6 myoblasts expressing chimeras consisting of approximately half GLUT1 and half GLUT4 (*upper panels*), and of approximately each quarter of GLUT4, with the remainder GLUT1 (*lower panels*). Shown in the upper panels are: 41-222 (junction C), 41-248 (junction D), 14-232 (junction D), and 14-271 (junction E). Shown in the lower panels are: 41-132 (junction B), 141-116,272 (junctions B and E), 141-271,360 (junctions E and F), and 14-359 (junction F). Antibodies include: F350, which recognizes the COOH terminus of GLUT1; F349, which recognizes the COOH terminus of GLUT4; and 37.6, the species-specific monoclonal antibody which recognizes an epitope of the GLUT1 intracellular loop engineered into most chimeras. Clonal lines: 41-248; 14-232; 14-271; and 41-132; pooled G418 resistant cells: 41-222; 141-116,272; 141-271,360; 14-359. Bars, 20 μm.

tail. Fig. 5 shows the targeting of 14-450 (junction H), which contains only the COOH-terminal cytoplasmic tail of GLUT4, using both the polyclonal antibody F349 and the monoclonal antibody 37.6. Targeting of this chimera, which contains only the final 43 amino acids of GLUT4, was indistinguishable from that of GLUT4 itself. Also shown in Fig. 5 are 41-440 (junction G) and 41-466 (junction H), which were targeted to the plasma membrane, as expected. Finally, 414-440,467 (junctions G and H), was sequestered within the cell. Taken together, these data show that the COOH-terminal cytoplasmic tail of GLUT4 is both necessary and sufficient to confer intracellular sequestration on chimeric transporters.

To further localize the targeting information within the COOH terminus, a series of constructs consisting of progressively more GLUT1 and less GLUT4 was designed. These include 14-463 (junction I), 14-468 (junction J), 14-473 (junction K), and 14-481 (junction L). Fig. 6 shows a striking difference between the intracellular targeting of 14-463 and 14-468, whose targeting was indistinguishable from GLUT4, and the targeting of 14-473 and 14-481, whose targeting resembled that of GLUT1. This difference is apparent both with the polyclonal antibody to GLUT4 and the species-specific monoclonal antibody to the intracellular loop of GLUT1. This suggests that the six amino acids of GLUT4 which differ between the constructs 14-468 and 14-473, RTPSLL, contain information that is necessary for the intracellular sequestration of GLUT4.

Of note in the six amino acid sequence RTPSLL is a dileucine motif, which has been shown to be an essential compo-

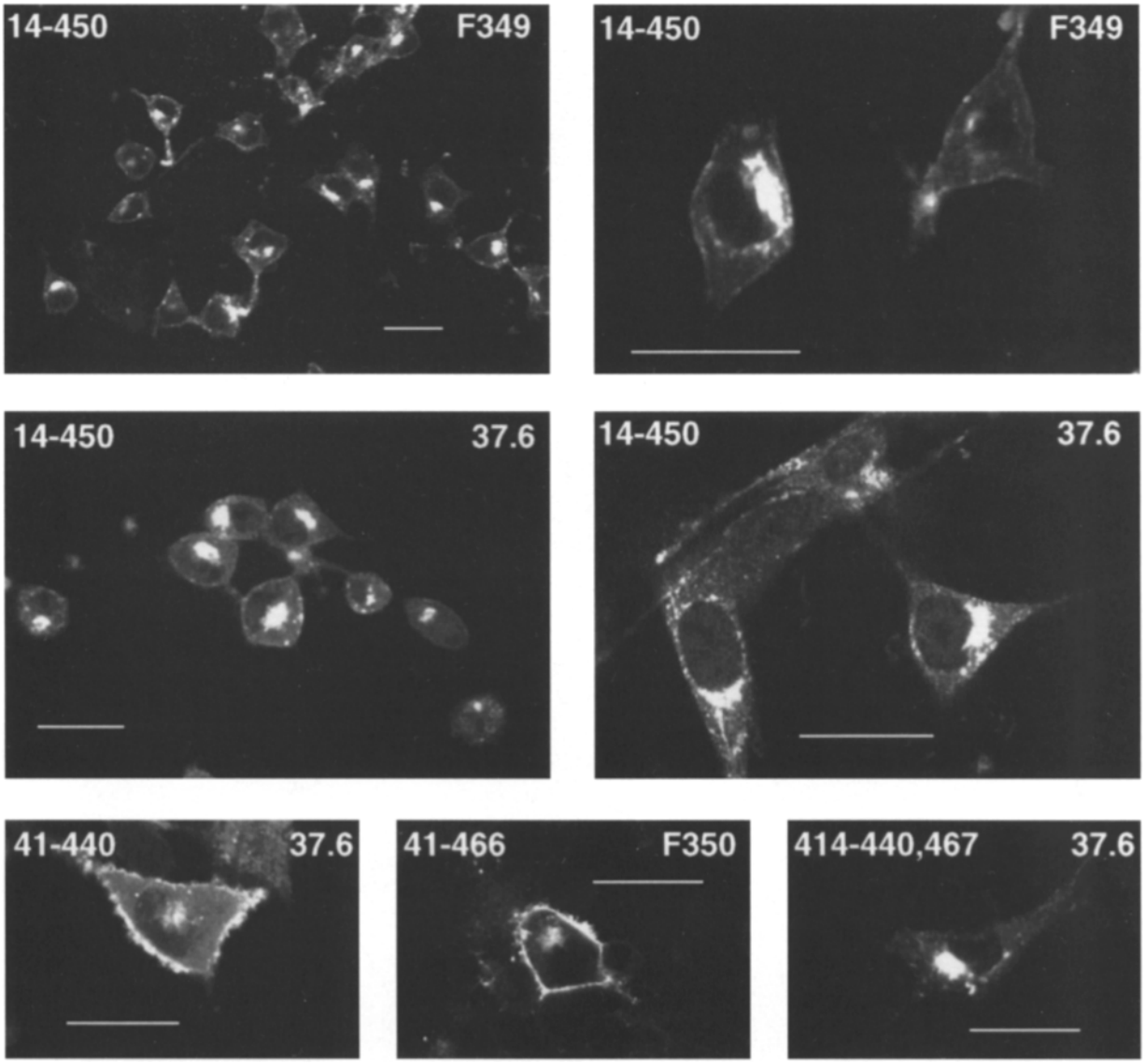

Figure 5. Confocal immunofluorescence microscopy of transfected L6 myoblasts expressing chimeras dissecting the intracellular targeting properties of the fourth quarter of GLUT4. Shown are: 14-450 (junction H), 41-440 (junction G), 41-466 (junction H), and 414-440,467 (junctions G and H). Antibodies include: F350, which recognizes the COOH terminus of GLUT1; F349, which recognizes the COOH terminus of GLUT4; and 37.6, the species-specific monoclonal antibody which recognizes an epitope of the GLUT1 intracellular loop engineered into most chimeras. Clonal lines: 14-450 and 41-440; pooled G418-resistant cells: 41-466 and 414-440,467. Bars, 20 μm.

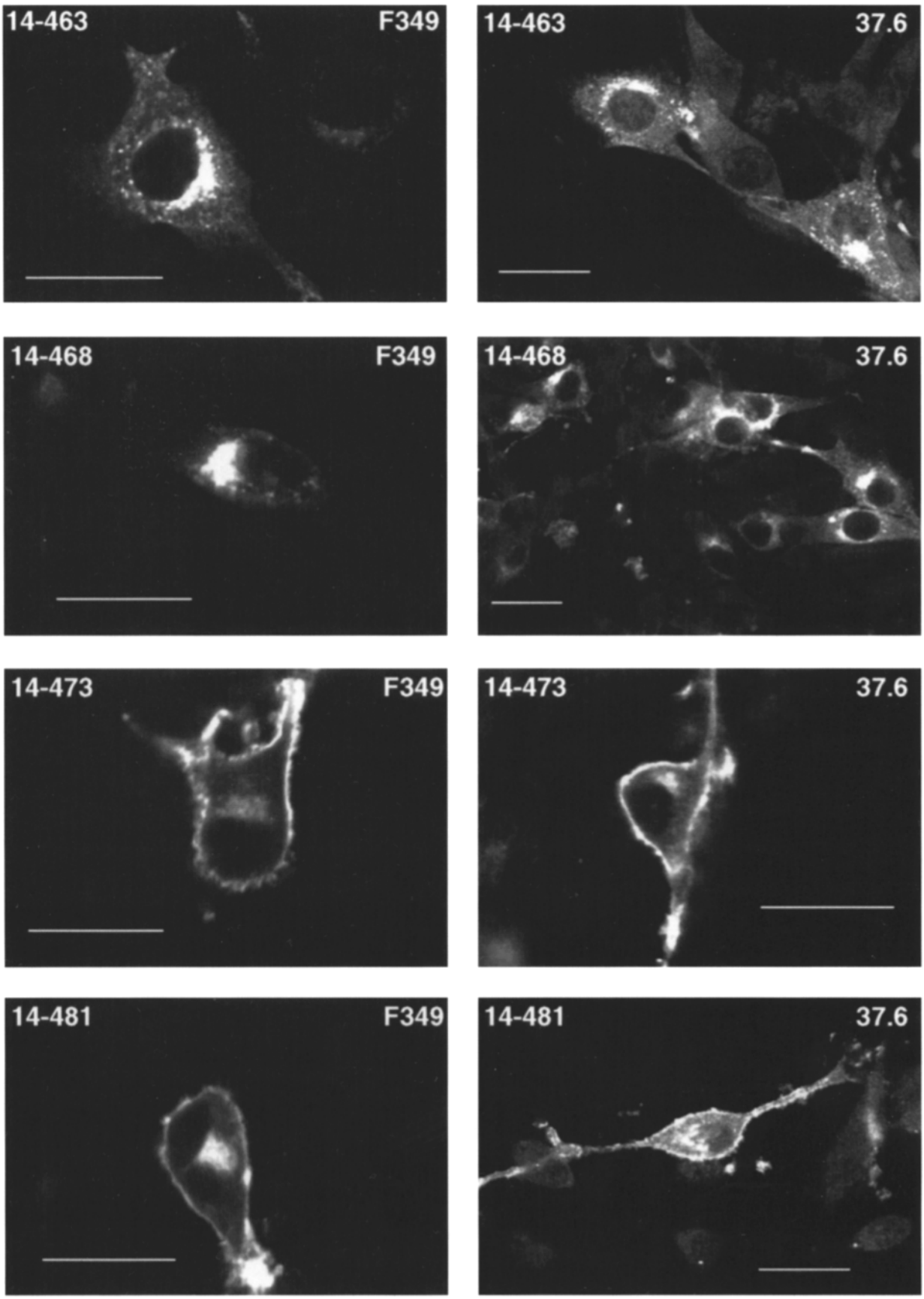

Figure 6. Confocal immunofluorescence microscopy of transfected L6 myoblasts expressing chimeras containing mostly GLUT1 and progressively smaller portions of the COOH-terminal cytoplasmic tail of GLUT4. Shown are: 14-463 (junction I), 14-468 (junction J), 14-473 (junction K), and 14-481 (junction L). Antibodies include: F349, which recognizes the COOH terminus of GLUT4, and 37.6, the species-specific monoclonal antibody which recognizes an epitope of the GLUT1 intracellular loop contained in these chimeras. Clonal line: 14-468; pooled G418-resistant cells: 14-463, 14-473, and 14-481. Bars, 20 μm.

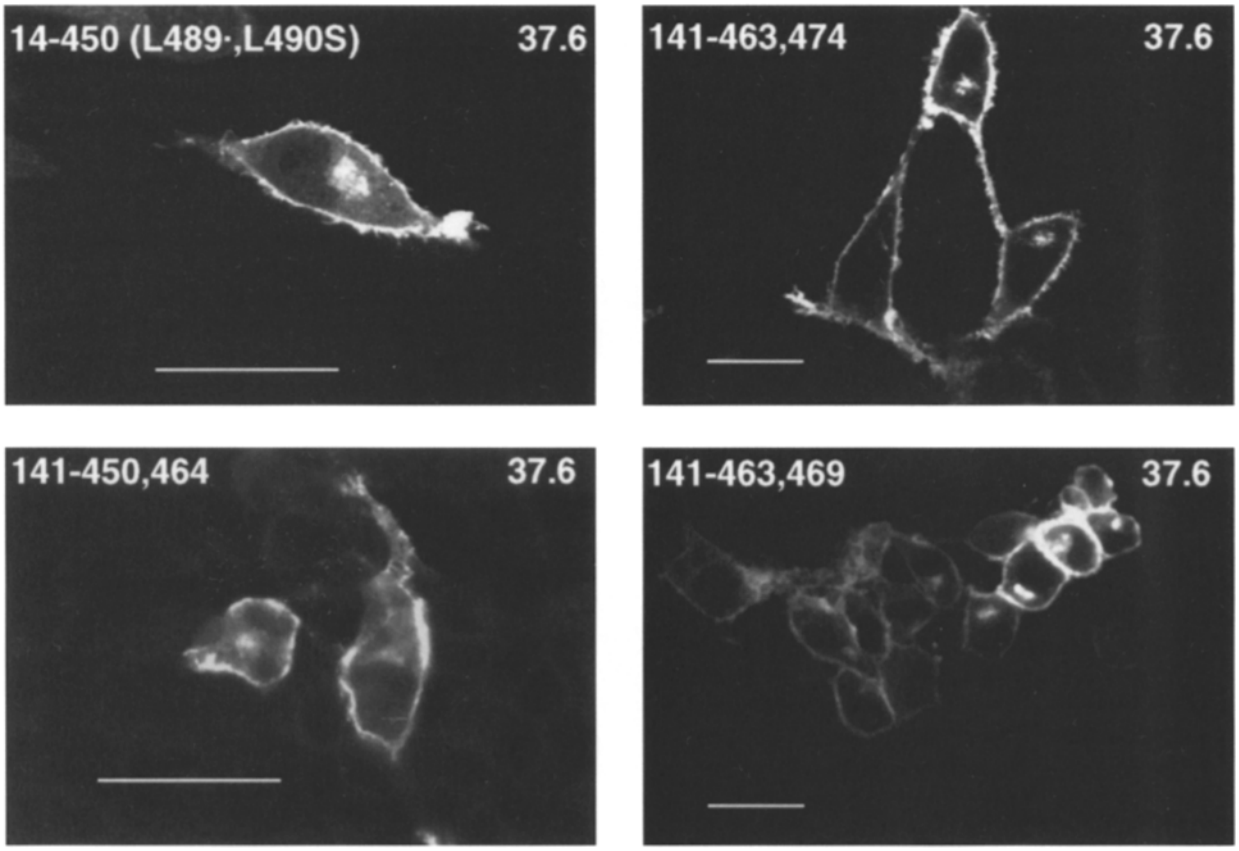

Figure 7. Confocal immunofluorescence microscopy of transfected L6 myoblasts expressing chimeras designed to dissect the intracellular targeting activity of the COOH-terminal cytoplasmic tail of GLUT4. Shown are: 14-450 (L489; L490S), 141-463, 474 (junctions I and K), 141-450, 464 (junctions H and I), and 141-463, 469 (junctions I and J). Cells were stained with 37.6, the species-specific monoclonal antibody which recognizes an epitope of the GLUT1 intracellular loop contained in these chimeras. Shown are pooled G418-resistant cells. Bars, 20 μm.

nent of certain targeting signals, and which has been reported to be important for GLUT4 targeting in insulin-insensitive cells (Verhey and Birnbaum, 1994). We designed the construct 14-450 (L489·, L490S) to test whether the dileucines are essential to the targeting of GLUT4; the region of GLUT1 analogous to the dileucine motif of GLUT4 contains only a single serine residue (Birnbaum, 1989; Charron et al., 1989; Fukumoto et al., 1989; James et al., 1989; Kaestner et al., 1989). As shown in Fig. 7, mutation of the dileucine motif to the corresponding amino acid (·S) of GLUT1 had a striking effect on the targeting of 14-450; the mutant construct demonstrated abundant plasma membrane staining. Targeting of the construct 141-463, 474 (junctions I and K), which exhibits a distribution similar to GLUT1, showed that neither the dileucine motif nor the six-amino acid sequence RTPSLL is sufficient to confer intracellular targeting, and that at least some of the amino acid residues beyond L490 of GLUT4 are required for intracellular targeting. The constructs 141-450, 464 (junctions H and I) and 141-463, 469 (junctions I and J) showed a distribution similar to GLUT1, confirming that the first 18 of the 43 amino acids of the COOH terminus are not sufficient to confer intracellular targeting.

To verify that the final 25 amino acid residues of GLUT4 are sufficient and the dileucine motif is essential for intracellular targeting, the constructs 41-479 (junction I), 41-490 (junction K) and 41-498 (junction L) were designed (Fig. 8). We predicted, based on the above results, that 41-479 and 41-490 would exhibit GLUT1-like targeting behavior, and that the targeting of 41-498 would depend on whether all of the remaining amino acids that are necessary to confer intracellular targeting lie between E491 and T498 of GLUT4. In contrast to our prediction, all three constructs demonstrated intracellular targeting (Fig. 8). This suggested that more than one mechanism might be involved in the intracellular targeting of glucose transporter chimeras. We designed the chimera 414-440,491 (junctions G and K) to analyze this further. This chimera lacks the dileucine motif and therefore would not be expected to confer intracellular targeting. The portions of it made up of GLUT4, the first 440 amino acids and the final 19 amino acids, were shown above to not cause intracellular targeting when tested independently, in chimeras 41-440 and 14-473. Paradoxically, as shown in Fig. 9, this chimera was retained within the cell and was excluded from the plasma membrane. It is demonstrated below that the intracellular accumulation of this chimera does not reflect targeting to an insulin-sensitive compartment.

Because of previous reports that the NH_2 terminus of GLUT4 confers intracellular sequestration (Piper et al., 1992, 1993), we examined the targeting of 41-24 (junction A), which contains the NH_2 terminus of GLUT4 with the remainder of the molecule being GLUT1. Fig. 9 shows that

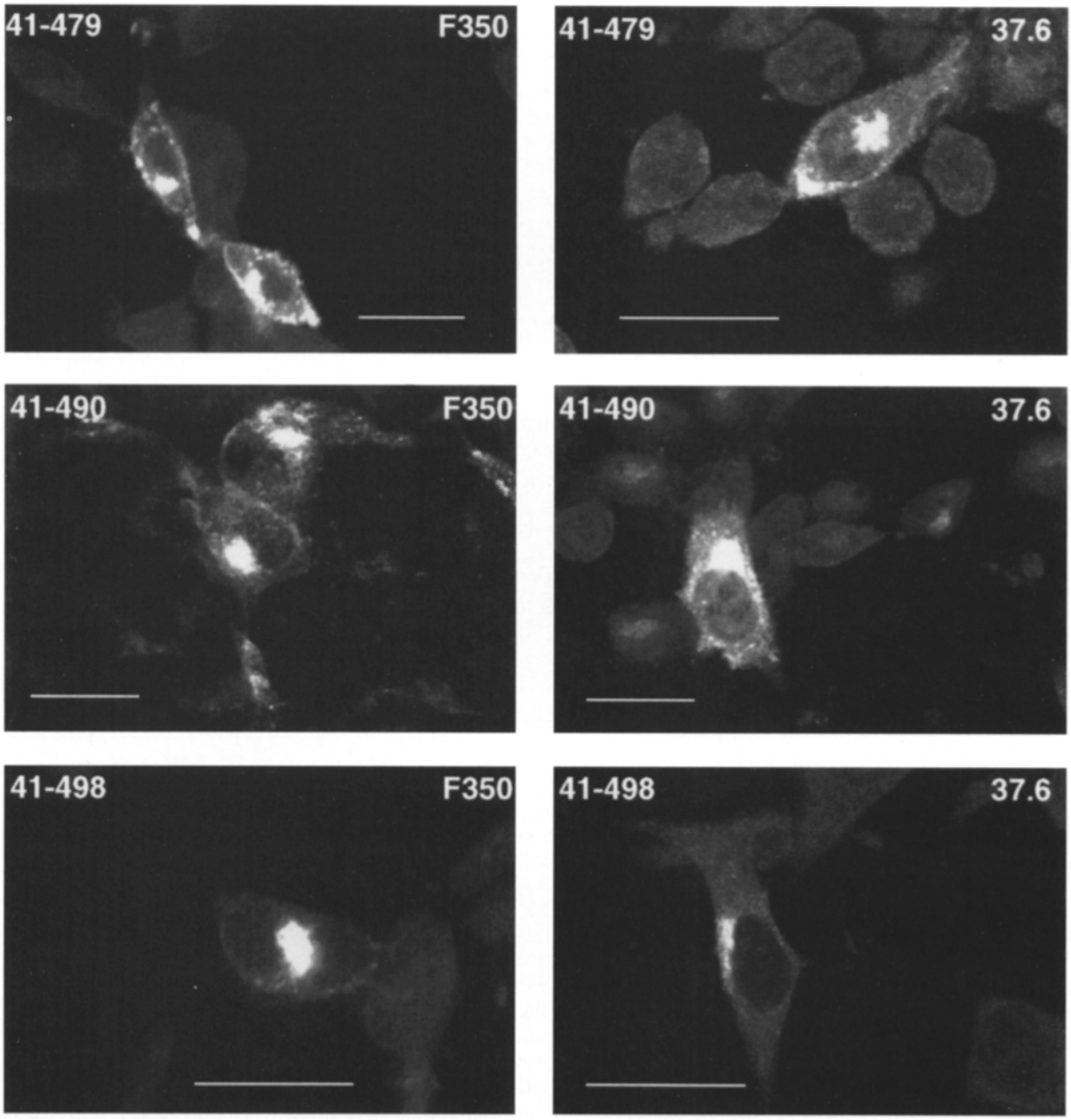

Figure 8. Confocal immunofluorescence microscopy of transfected L6 myoblasts expressing chimeras containing mostly GLUT4 and progressively smaller portions of the COOH-terminal cytoplasmic tail of GLUT1. Shown are: 41-479 (junction I), 41-490 (junction K), and 41-498 (junction L). Antibodies include: F350, which recognizes the COOH terminus of GLUT1, and 37.6, the species-specific monoclonal antibody which recognizes an epitope of the GLUT1 intracellular loop engineered into these chimeras. Shown are pooled G418-resistant cells. Bars, 20 μm.

this construct exhibited targeting indistinguishable from GLUT1. In contrast, 414-24,467 (junctions A and H), which contains the NH_2 and COOH termini of GLUT4 flanking GLUT1 sequence, showed targeting indistinguishable from GLUT4.

Our goal was to study chimeric glucose transporter targeting in an insulin-responsive system. One method to evaluate insulin-responsive targeting is by quantitating transporters in partially purified fractions of plasma membrane and intracellular membranes. We carried out preliminary experiments using published procedures but concluded, as have others (Lawrence et al., 1992), that these procedures did not result in a useful and reproducible degree of enrichment. Attempts to demonstrate changes in the subcellular distribution of GLUT4 in response to insulin with confocal immunofluorescence microscopy were also unconvincing. We therefore examined whether immunogold labeling and electron microscopy might be more sensitive for detection of translocation. Slot et al. have shown the usefulness of this method for detection of GLUT4 translocation in brown adipose tissue

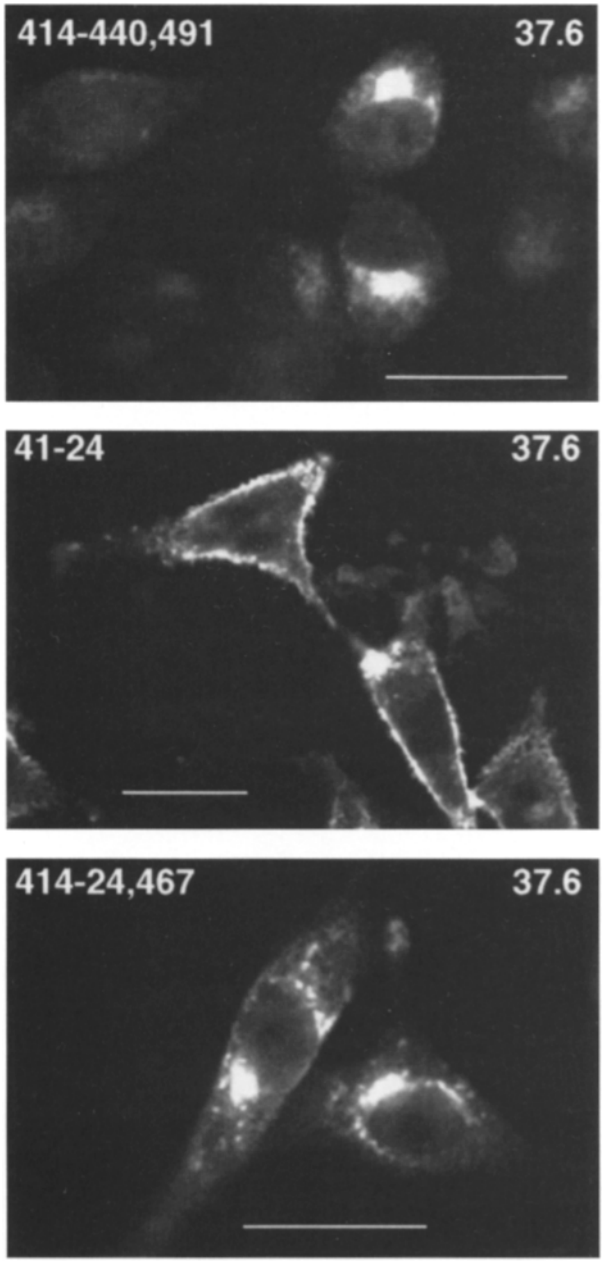

Figure 9. Confocal immunofluorescence microscopy of transfected L6 myoblasts. Shown are: 414-440, 491, which contains two portions of GLUT4, the first 440 amino acids and the final 19 amino acids; 41-24 (junction A), which contains the NH_2 terminus of GLUT4; and 414-24, 467 (junctions A and H), which contains the NH_2 and COOH termini of GLUT4. Cells were stained with 37.6, the species-specific monoclonal antibody which recognizes an epitope of the GLUT1 intracellular loop contained in these chimeras. Shown are clonal cell lines. Bars, 20 μm.

(1991*a*) and in cardiac myocytes (1991*b*). GLUT1 staining was abundant in the plasma membrane, especially in microvilli (Fig. 10). In contrast, GLUT4 labeling of the plasma membrane was minimal and staining was concentrated in endosome-like vesicles and tubulovesicular structures, often in close proximity to Golgi stacks (Fig. 11). Thus, the distribution of GLUT4 in L6 myoblasts was very similar to that reported in brown fat (Slot et al., 1991*a*), skeletal muscle (Rodnick et al., 1992), and cardiac myocytes (Slot et al., 1991*b*). Except for GLUT4, all quantitation was done using the species-specific monoclonal antibody 37.6, which recognizes only the exogenous transporter. Independent clonal cell lines were studied to eliminate clonal variability as a possible explanation for the results and to exclude the possibility of systematic error. Under basal conditions, plasma membrane staining accounted for approximately half of GLUT1 but only about 5% of GLUT4 (Table I). Treatment with insulin did not affect the basal GLUT1 labeling pattern but caused a 3.7–6.1-fold increase in the proportion of GLUT4 in the plasma membrane. The chimera 14-450 (junction H), which contains only the COOH-terminus of GLUT4, was just as effectively sequestered within the cell as GLUT4, showing only ~5% of the signal in the plasma membrane. After treatment with insulin, the proportion of the labeling in the plasma membrane increased by 6.8–10.5-fold. Fig. 12 shows an example of the labeling pattern observed for 14-450 in the presence and absence of insulin. The pattern of its staining was very similar to that of GLUT4 under both conditions. The chimera 41-24 (junction A), which contains only the NH_2 terminus of GLUT4, targeted similarly to GLUT1. The chimera 414-440,491, which paradoxically exhibited intracellular targeting, showed a result intermediate between GLUT1 and GLUT4, with 14.5–19.7% of the labeling in the plasma membrane. However, no redistribution of the labeling occurred in response to insulin. Thus, this chimera appears to be targeted to intracellular compartments that are not insulin responsive.

The function of chimeric glucose transporters was examined by measurement of basal and insulin-stimulated 2-deoxyglucose uptake in clonal cell lines expressing them. Interpretation of these data is hindered by the heterogeneity of endogenous GLUT1 and GLUT3 expression in the clonal lines and by the heterogeneity of expression of the chimeras themselves. Nevertheless, three conclusions can be drawn from the results shown in Table II. First, overexpression of GLUT1 and of the chimera containing only the NH_2 terminus of GLUT4, 41-24 (junction A), elevated basal glucose transporter activity, as would be expected for transporters targeted to the plasma membrane. Second, most cell lines exhibited the degree of insulin responsiveness characteristic of L6 myoblasts, that is, about a 1.3- to 1.5-fold stimulation of 2-deoxyglucose uptake. In contast to the report of Robinson et al. (1993), L6 cells expressing GLUT4 did not show an increase in insulin responsiveness. It is important to note that the L6 cells studied by Robinson et al. (1993) were actually a subclone of L6 cells, which they acknowledged behaved abnormally and did not express glucose transporters in a pattern consistent with previous studies of L6 cells. The L6 subclone studied by Robinson et al. (1993) expressed extremely low levels of endogenous GLUT1 and GLUT3 compared to other reports (Bilan et al., 1992; Mitsumoto and Klip, 1992) and to the cells we studied. Robinson et al. also expressed GLUT4 at up to 30-fold higher levels than in untransfected cells, while expression of GLUT4 in the cells we studied was limited to eightfold above the level in untransfected cells. In other words, GLUT4 was the predominant glucose transporter in the cells of Robinson et al., but only a minor glucose tranporter in the cells we studied. However, the cell line

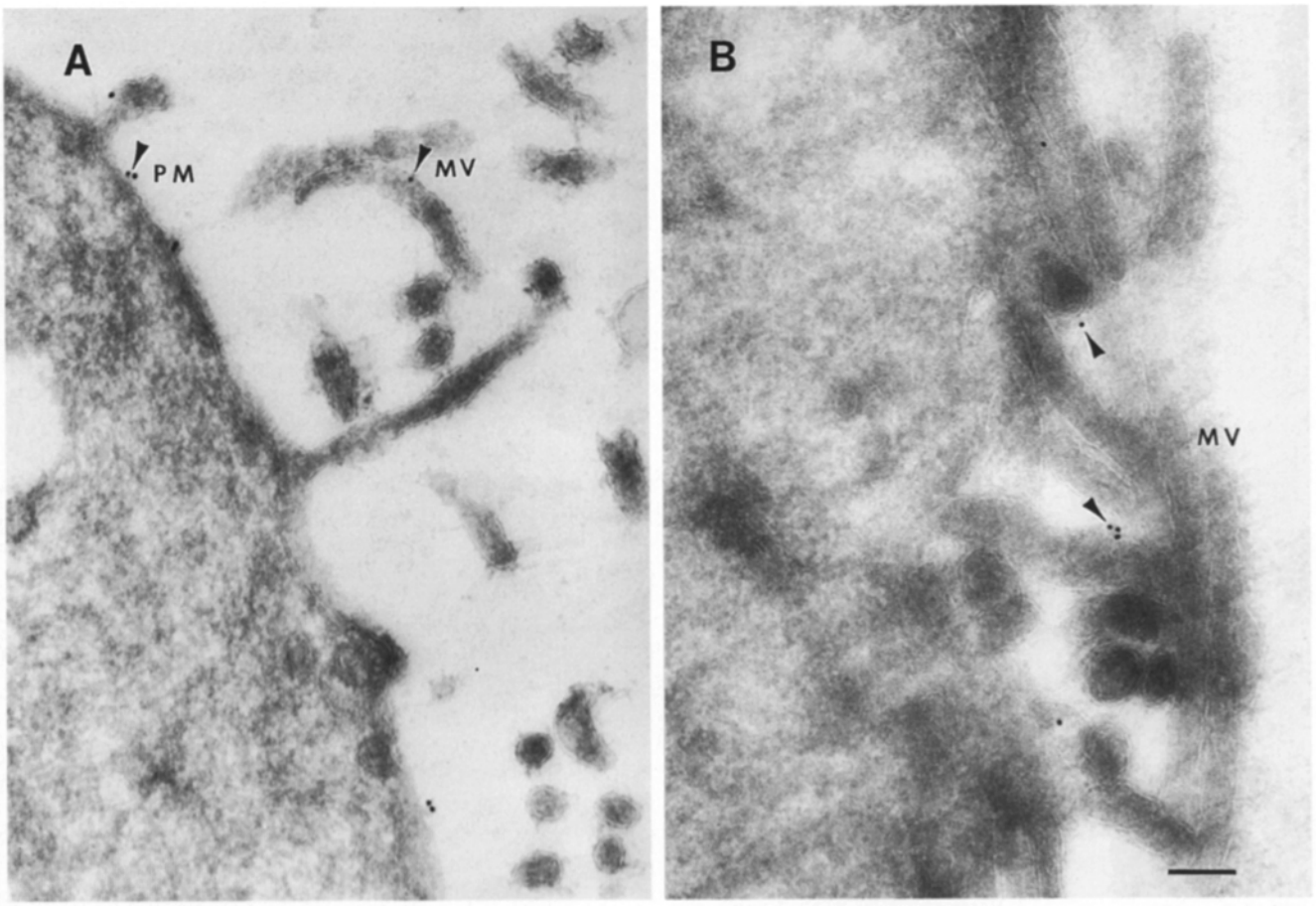

Figure 10. Ultrastructural localization of GLUT1 in transfected L6 myoblasts. Frozen thin sections of L6 myoblasts overexpressing GLUT1 were stained with F350, followed by goat anti-rabbit IgG complexed to 18-nm gold particles. Cells are shown under basal conditions (*A*) and after exposure to insulin (*B*) as described in Materials and Methods. Under both conditions, GLUT1 is enriched in plasma membrane domains, especially microvilli. *PM*, plasma membrane; *MV*, microvilli. Bar, 175 nm.

expressing the chimera containing only the COOH terminus of GLUT4, 14-450 (junction H), responded to insulin with a 2.7-fold increase in transport activity. Detection of insulin responsiveness by measurement of 2-deoxyglucose uptake in these cells was possible because, compared to the cells we studied that overexpressed GLUT4, cells expressing 14-450 contain 60% less GLUT1, 20% less GLUT3, and express three times as much of a glucose transporter that appears to translocate more efficiently than GLUT4 itself. Expression of this chimera did not appreciably alter basal 2-deoxyglucose uptake. Finally, the cells expressing the chimera which exhibited paradoxical intracellular targeting, 414-440,491 (junctions G and K), exhibited no insulin responsiveness, but showed very high basal 2-deoxyglucose uptake. This was most likely due to the high levels of expression of endogenous GLUT1 and GLUT3 in this cell line.

Discussion

As reviewed in the introduction, several groups have studied GLUT4 targeting domains in insulin-insensitive cells and have reported contradictory results. There are several possible explanations for discrepancies in identification of GLUT4 targeting domains. The finding that transgenic mice overexpressing GLUT4 in fat (Shepherd et al., 1993) and muscle (Liu et al., 1993) do demonstrate changes in basal as well as insulin-stimulated glucose uptake raises the possibility that certain levels of expression of GLUT4 saturate the machinery responsible for its accurate sorting and intracellular sequestration. In the study of Piper et al. (1992), as pointed out previously (Asano et al., 1992), chimeric transporters were expressed at twice the level of expression of GLUT4 in 3T3-L1 adipocytes. Furthermore, this was achieved by lethally infecting cells with recombinant Sindbis virus and subsequently treating the cells with cycloheximide. Asano et al. (1992) and Czech et al. (1993) have commented on the potential problems with this method. To test the validity of this approach, we carried out preliminary experiments using recombinant Vaccinia virus, which also lethally infects cells and causes high levels of expression of exogenous proteins. We performed these experiments in several different cell types, including L6 myoblasts, 3T3-L1 fibroblasts and adipocytes, rat1 fibroblasts, and CV1 cells. We achieved high levels of expression in all cell types, and noted both GLUT1 and GLUT4 to be distributed both to the plasma membrane and to intracellular compartments (data not shown). Targeting patterns varied according to the time elapsed after infection, and the reproducibility was poor. We speculate that specific sorting mechanisms are saturated and overwhelmed at the levels of expression of chimeric transporters caused by

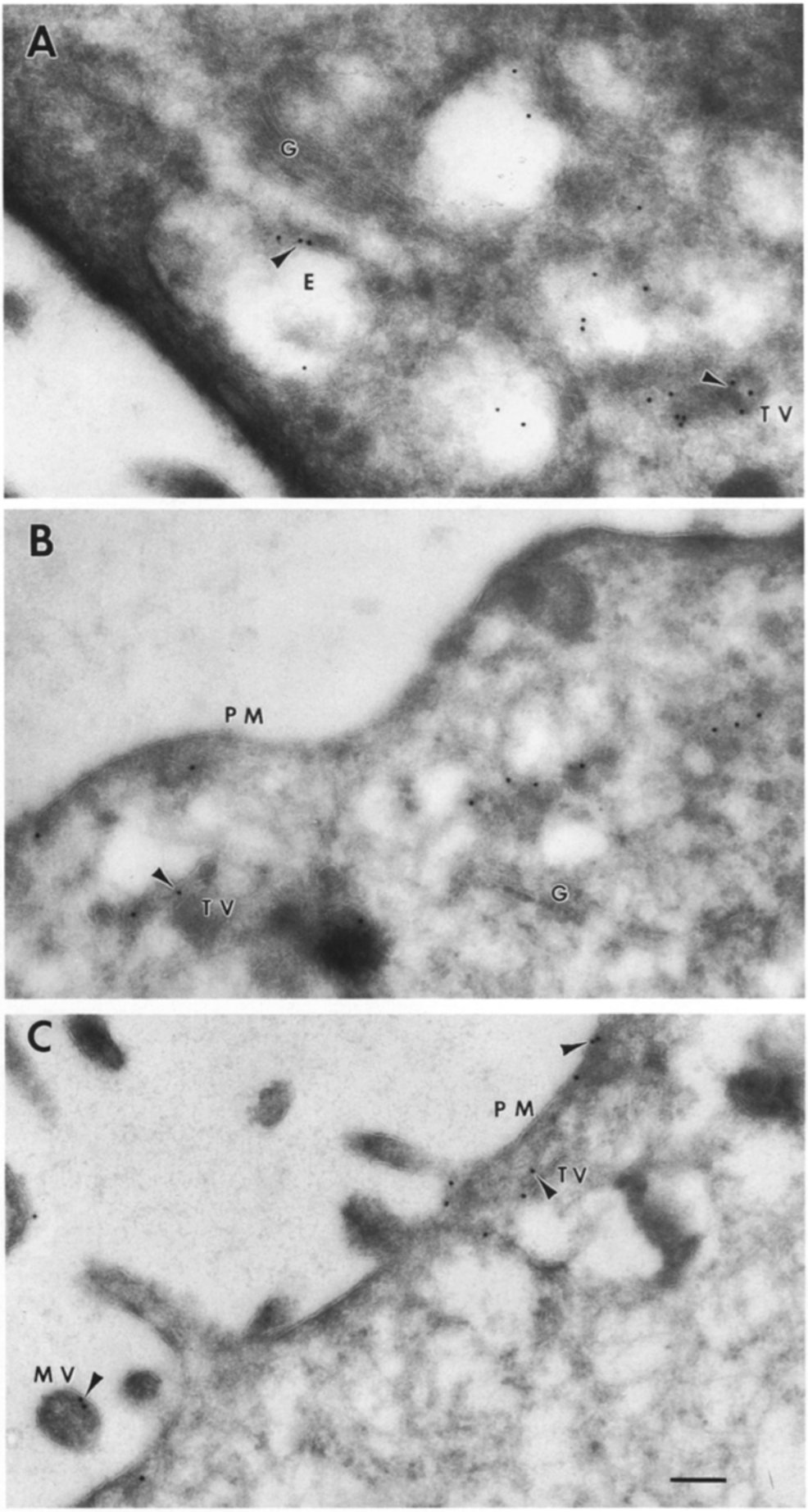

Figure 11. Ultrastructural localization of GLUT4 in transfected L6 myoblasts. Frozen thin sections of L6 myoblasts overexpressing GLUT4 were stained with F349, followed by goat anti-rabbit IgG complexes to 18-nm gold particles. Cells are shown under basal conditions (*A* and *B*) and after exposure to insulin (*C*) as described in Materials and Methods. Staining of GLUT4 in basal cells is concentrated in endosome-like compartments and tubulovesicular structures, often in close proximity to Golgi stacks (*A*). GLUT4 labeling of the plasma membrane is minimal in basal cells (*B*). The GLUT4 labeling of the plasma membrane increased after insulin treatment (*C*). *PM*, plasma membrane; *MV*, microvilli; *TV*, tubulo-vesicular elements; *E*, endosome-like compartment; *G*, Golgi. Bar, 175 nm.

Table I. Distribution of Wild-type and Chimeric Glucose Transporters in L6 Myoblasts

Expressed protein	Clonal line	Condition	Proportional distribution		Particles per cell
			Plasma membrane	Intracellular	
GLUT1	61	Basal	51.4 ± 1.6	48.6 ± 1.6	24.8 ± 3.1
		Insulin	55.2 ± 0.8	44.8 ± 0.8	46.7 ± 3.6
	67	Basal	53.1 ± 1.6	46.9 ± 1.6	21.5 ± 2.1
		Insulin	56.9 ± 5.3	43.1 ± 5.3	22.4 ± 3.1
GLUT4	35	Basal	6.1 ± 1.0	93.9 ± 1.0	27.7 ± 2.5
		Insulin	24.4 ± 2.7	76.6 ± 2.7	31.2 ± 2.7
	44	Basal	5.2 ± 1.3	94.8 ± 1.3	22.9 ± 6.9
		Insulin	19.3 ± 1.8	80.7 ± 1.8	49.6 ± 5.8
	83	Basal	4.2 ± 1.1	95.8 ± 1.1	38.3 ± 3.4
		Insulin	25.5 ± 2.0	74.5 ± 2.0	37.7 ± 2.8
14-450	36	Basal	5.2 ± 0.9	94.8 ± 0.9	31.6 ± 3.8
		Insulin	35.2 ± 2.9	64.8 ± 2.9	28.7 ± 2.8
	63	Basal	4.6 ± 1.8	95.4 ± 1.8	32.5 ± 3.0
		Insulin	48.5 ± 1.4	51.5 ± 1.4	51.8 ± 6.1
	93	Basal	4.8 ± 1.1	95.2 ± 1.1	27.6 ± 2.0
		Insulin	41.7 ± 2.7	58.3 ± 2.7	23.5 ± 1.8
41-24	36	Basal	60.1 ± 1.5	39.9 ± 1.5	26.2 ± 2.3
		Insulin	56.1 ± 1.1	43.9 ± 1.1	34.7 ± 2.6
414-440,491	51	Basal	14.5 ± 0.9	85.5 ± 0.9	50.6 ± 6.3
		Insulin	13.4 ± 1.1	86.6 ± 1.1	60.6 ± 3.7
	71	Basal	19.7 ± 1.1	80.3 ± 1.1	38.6 ± 2.8
		Insulin	17.9 ± 1.2	82.1 ± 1.2	42.0 ± 2.4

Ultra-thin cryosections of transfected L6 myoblast lines were prepared, stained by indirect immunogold labeling, and analyzed as described in Materials and Methods. The species-specific monoclonal antibody 37.6 was used for quantitation in all cases except for cells overexpressing GLUT4, which were quantitated using F349. Results are shown as means ± SEM for 10 cells per condition per clonal line. Untransfected cells showed a range of 0 to 4 particles per cell, primarily within the nucleus. Particles staining the nucleus constituted no more than 15% of the staining in transfected cells and were not included in calculations of proportional distribution. For certain clones, apparent differences in particles per cell between basal and insulin-stimulated states may not be meaningful because the two conditions were not studied side by side. These include: GLUT1, clone 61; GLUT4, clone 44; 14-450, clone 63; 41-24, clone 36; and 414-440,491, clone 51. The remaining clones were studied side by side.

recombinant Sindbis virus and Vaccinia virus. Therefore, we studied chimeras expressed in stably transfected L6 cells. The CMV-based expression vector we used resulted in expressin of heterologous transporters at no higher an abundance than that of endogenous transporters, addressing concerns related to saturation of a cellular targeting mechanism. This permitted examination of the subcellular distribution of chimeras expressed in a steady-state at relatively low levels. We and others consider this condition an absolute prerequisite for valid targeting studies; Czech et al (1993) offered a model for GLUT4 targeting in which the specific GLUT4 targeting mechanism was saturated in the range of total endogenous transporter expression. One unavoidable consequence of our approach, especially considering the levels of GLUT1 (Mitsumoto and Klip, 1992) and GLUT3 (Bilan et al., 1992) expression in L6 cells, is that the expression of relatively low levels of heterologous transporters can not be expected to result in several-fold changes in total cellular glucose uptake. Certain subclonal L6 cell lines overexpressing GLUT4 (Lawrence et al., 1992; Robinson et al., 1993) contain much less GLUT1 and GLUT3 than unselected L6 cells and are capable of exhibiting a several-fold stimulation of glucose uptake in response to insulin. However, in the cells we studied, which show abundant expression of GLUT1 and GLUT3, subcellular targeting behavior can not be inferred from changes in glucose transport activity alone.

A further complication in interpreting the study of Piper et al. (1992) is that the chimeras used to demonstate intracellular targeting presumably conferred by the NH_2 terminus also contained that portion of the COOH terminus which others have shown is important in intracellular sequestration (Czech et al., 1993; Marshall et al., 1993*a*; Verhey et al., 1993; this study). It is especially puzzling that Piper et al. did not detect the intracellular sequestration caused by the COOH terminus in either their original report (1992) or in a subsequent study (Piper et al., 1993*a*) attempting to define the NH_2-terminal targeting motif.

Immunocytochemical data from confocal immunofluorescence microscopy (Fig. 9) and immunogold electron microscopy (Table I), as well as 2-deoxyglucose uptake (Table II), demonstrate that the NH_2 terminus of GLUT4 has no discernible intracellular targeting activity in L6 myoblasts. Fig. 9 also shows that a chimera similar to the constructs used by Piper et al. (1992), 414-24, 467, does indeed display intracellular targeting, but this is attributable to its COOH terminus, not its NH_2 terminus. Garippa et al. (1994) suggest that the NH_2 terminus of GLUT4 does not function as an intracellular retention signal but rather contains an internalization motif. However, chimeras of this motif and the transferrin receptor show a ~50% localization at the cell surface (Garippa et all, 1994). These results clearly rule out an important role for this motif in causing the virtual exclusion of

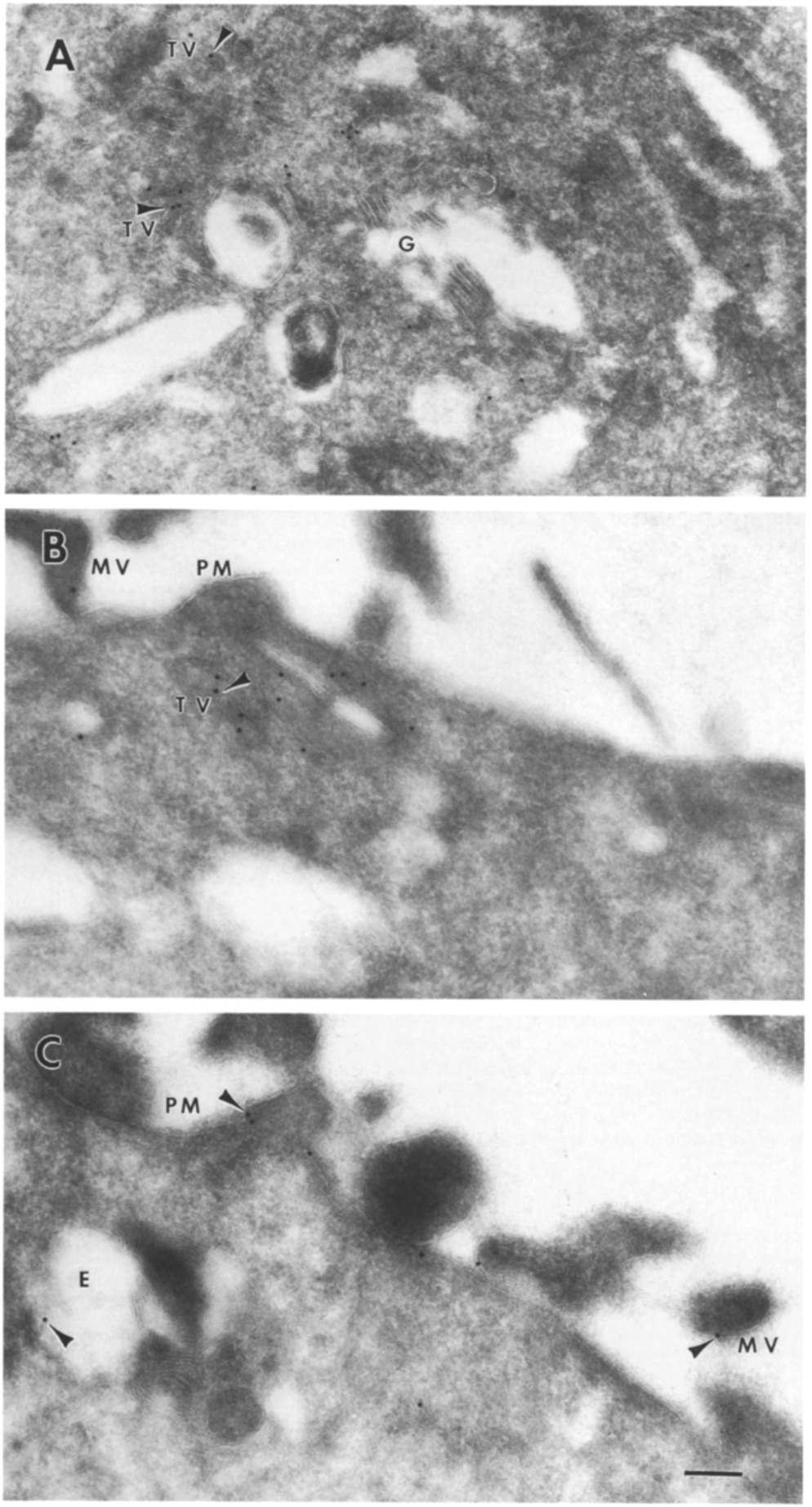

Figure 12. Ultrastructural localization of 14-450 (junction H), which contains the COOH-terminal cytoplasmic tail of GLUT4, in transfected L6 myoblasts. Its staining pattern was very similar to that of GLUT4 (see Fig. 11). Cells are shown under basal conditions (*A* and *B*) and after exposure to insulin (*C*) as described in Materials and Methods, stained with F349. The plasma membrane labeling increased by 6.8–10.5-fold after insulin treatment. *PM*, plasma membrane; *MV*, microvilli; *TV*, tubulovesicular elements; *E*, endosome-like compartment; *G*, Golgi. Bar, 175 nm.

Table II. 2-Deoxyglucose Uptake of L6 Myoblasts Expressing Wild-type and Chimeric Glucose Transporters

	Endogenous GLUT1	Endogenous GLUT3	Total GLUT4	Heterologous transporter	Basal 2-DOG uptake	Insulin-stimulated 2-DOG uptake	Insulin-responsiveness
	ng/20 μg	*L6 = 1.0*	*ng/20 μg*	*ng/20 μg*	*pmol/mg/min*	*pmol/mg/min*	*fold*
L6	6.8	1.0	0.2	0	124 ± 12	170 ± 22	1.4
GLUT1	4.3	0.8	0.2	13.1	232 ± 42	303 ± 21	1.3
GLUT4	9.2	1.5	1.6	nd	144 ± 8	205 ± 12	1.4
14-450	3.8	1.2	0.5	5.0	146 ± 6	400 ± 82	2.7
41-24	8.5	1.5	0.6	5.2	198 ± 29	306 ± 29	1.5
414-440,491	20.2	2.5	0.5	3.3	296 ± 42	294 ± 38	1.0

The content of endogenous and exogenous transporters was determined by densitometric analysis of Western blots as described in Materials and Methods. The content of the heterologously expressed chimeric transporters was measured using the species-specific monoclonal antibody 37.6. Polyclonal antibodies against the COOH termini of GLUT1 and GLUT4 recognized both endogenous and chimeric transporters. For the cell lines expressing GLUT1 and 41-24, the content of endogenous GLUT1 was calculated by subtracting the content of the chimeric transporter from the transporters recognized by the polyclonal antibody F350. For cell lines expressing GLUT4, 14-450, and 414-440,491, the total GLUT4 content was calculated by subtracting the content of the chimeric transporter from the transporters recognized by the polyclonal antibody F349. GLUT4 does not contain the epitope recognized by the monoclonal antibody, so that a value for the heterologous transporter content cannot be determined (*nd*, not determined). The GLUT3 content is expressed relative to the GLUT3 content of untransfected cells. 2-Deoxyglucose uptake was measured as described in Materials and Methods. Cells were rinsed and maintained in serum-free medium for 2 h before determination of uptake over a 6-min period. Insulin treatment was for 20 min at 10^{-6} M. Results are shown as means ± SEM of six determinations and are corrected for non-carrier-mediated uptake as determined in the presence of 20 μM cytochalasin B.

GLUT4 from the plasma membrane of adipocytes and muscle cells. Neither our data (Marshall et al., 1993*a*; this report) nor those published by other investigators (Asano et al., 1992; Czech et al., 1993; Verhey et al., 1993, 1994) support the hypothesis of Piper et al. (1992, 1993) that the NH_2-terminal cytoplasmic tail of GLUT4 is important in determining its steady-state distribution.

Three of the previous studies of GLUT4 targeting domains in non–insulin-sensitive cells indicate that intracellular localization is a function not only of the COOH-terminal cytoplasmic tail but also a weaker determinant in the NH_2-terminal half of GLUT4 exclusive of the NH_2 terminus (Verhey et al., 1993; Czech et al., 1993; Marshall et al., 1993*a*). A targeting domain between amino acids 53 and 132 would be consistent with all three studies, as well as with one of the two signals identified by Asano et al. (1992). Nevertheless, in L6 cells, chimeras containing this region of GLUT4, but not the COOH-terminus, show targeting that appears to be very similar to GLUT1. Marshall et al. (1993*a*) presented evidence that chimeras containing this region of GLUT4 might be poorly transported beyond the endoplasmic reticulum in *Xenopus* oocytes. One possible explanation for this apparent discrepancy is that L6 cells may contain cell-type specific chaperone proteins that facilitate maturation of chimeric transporters containing this region of GLUT4. Intracellular targeting attributed to this region in studies of insulin-insensitive cells may reflect lack of such a chaperone protein.

We identified the region between amino acids 485 and 490 of GLUT4, RTPSLL, as necessary but not sufficient for GLUT4 targeting. This region is of interest not only because it contains a dileucine motif which is itself necessary for GLUT4 targeting (Verhey and Birnbaum, 1994), but also because it contains ser-488, the only known phosphorylation site of GLUT4 (Lawrence et al., 1990). This suggests the possibility that hormonally mediated protein phosphorylation-dephosphorylation plays a role in regulating the intracellular targeting activity of the domain. GLUT1 contains neither the dileucine motif nor an analogous phosphorylation site. Evidence from L6 cells suggests that cAMP specifically inhibits glucose transport mediated by GLUT4 but not by GLUT1 (Lawrence et al., 1992). Phosphorylation of ser-488 may reduce GLUT4 exocytosis, increase GLUT4 endocytosis, or redirect its intracellular trafficking. However, the relationship between GLUT4 phosphorylation and function is unclear (Nishimura et al., 1991; Piper et al., 1993*b*) and further experiments are necessary to examine this question.

Our data indicate that the final 25 amino acids of GLUT4 are necessary and sufficient to confer intracellular targeting. It remains to be determined whether there is more than one motif contained in this sequence. The COOH-terminal tail of the mannose-6-phosphate receptor contains two independent sorting motifs. One is a tyrosine-containing internalization signal required for rapid endocytosis from the plasma membrane via clathrin-coated pits. The second, the COOH-terminal four amino acids, includes a dileucine motif, and is required for efficient interaction with the Golgi adaptors (Johnson and Kornfeld, 1992*b*). The T cell antigen receptor gamma chain also contains two distinct sequences in its COOH-terminal cytoplasmic tail, a tyrosine-containing motif and a dileucine-containing motif required for appropriate intracellular targeting to lysosomes (Letourneur and Klausner, 1992). The dileucine motif has also been implicated in the intracellular targeting via Golgi to lysosomes of LIMP II, a lysosomal membrane protein (Vega et al., 1991; Ogata and Fukuda, 1994; Sandoval et al., 1994), the cation-dependent mannose-6-phosphate receptor (Johnson and Kornfeld, 1992*a*), and CD4, the main HIV receptor (Aiken et al., 1994). Verhey and Birnbaum (1994) have reported that the dileucine motif is required for GLUT4 targeting in fibroblasts; their cogent argument that GLUT4, not GLUT1, is the actively targeted transporter, and that the dileucines constitute an essential part of the GLUT4 COOH-terminal targeting motif, is supported by our findings in insulin-sensitive cells.

The steady state distribution of GLUT4 in the presence of insulin included 19.3–25.5% in the plasma membrane, a 3.7–6.1-fold enrichment compared to basal conditions. Of note, in the presence of insulin, the steady state distribution of the chimera 14-450, which contains only the COOH-terminal cytoplasmic tail of GLUT4, included 35.2–48.5% in the plasma membrane, a 6.8–10.5-fold enrichment com-

pared to basal conditions. Thus, the chimera seems to respond to insulin better than GLUT4 itself does. Since the steady state distribution reflects a balance between exocytosis and endocytosis, this suggests that, in the presence of insulin but not under basal conditions, the chimera 14-450 is either exocytosed more rapidly, endocytosed more slowly, or both. Our experimental approach does not distinguish between these mechanisms.

There is evidence that GLUT1 may oligomerize (Pessino et al., 1991), and that oligomerization may have important functional consequences (Hebert and Carruthers, 1992). This potential complication must be taken into account in interpretation of the targeting of chimeric glucose transporters. The area identified by this approach might theoretically represent not a targeting domain itself, but an area of the chimera that causes oligomerization with endogenous GLUT4, which contains the actual domains responsible for the intracellular targeting. Oligomerization of chimeras with endogenous GLUT1 or GLUT3 would presumably not cause misidentification of an intracellular targeting motif, since these transporters are not sequestered within the cell. Oligomerization is itself a potential mechanism for retention of Golgi-resident proteins (Weisz et al., 1993). The distinct targeting patterns of GLUT1 and GLUT4 suggest that there is no interaction between these two isoforms. Coimmunoprecipitation experiments have shown that GLUT1 and GLUT4 do not interact, but that GLUT1 forms homo-oligomers (Pessino et al., 1991). Oligomerization of GLUT4 segments of chimeras with endogenous GLUT4 is not a plausible explanation for the intracellular targeting observed in our study. The cell lines expressing the chimera 14-450 contain at least ten times as much chimera as they do endogenous GLUT4. Therefore, it would be stoichiometrically impossible to attribute the intracellular targeting of this chimera to an association with endogenous GLUT4.

The high degree of homology (65% identity and 76% similarity) between GLUT1 and GLUT4 make the study of chimeric glucose transporters especially attractive. However, the aberrant intracellular targeting of 414-440, 491, and, presumably, of 41-479 (junction I) and 41-490 (junction K), amplifies the questions raised by Low et al. (1994) concerning the validity of using intracellular sequestration alone as an assay for specific targeting signals. Studies should be designed to examine redundant chimeras and converse chimeras; these offer protection against misidentifying targeting information. Specifically with respect to glucose transporters, a method that examines the insulin responsiveness of putative targeting domains is required to exclude aberrant targeting. In general, methods to establish not only the structural but also the physiological properties of a particular compartment should be required to establish the validity of putative targeting signals.

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