Distinct Reading of Different Structural Determinants Modulates the Dileucine-mediated Transport Steps of the Lysosomal Membrane Protein LIMPII and the Insulin-sensitive Glucose Transporter GLUT4*

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Leucine-based motifs mediate the sorting of membrane proteins at such cellular sites as the trans-Golgi network, endosomes, and plasma membrane. A Leu paired with a second Leu, Ile, or Met, while itself lacking the ability to mediate transport, is the key structural feature in these motifs. Here we have studied the structural differences between the leucine-based motifs contained in the COOH tails of LIMPII and GLUT4, two membrane proteins that are transported through the secretory pathway and are targeted to lysosomes (1–3) and to a perinuclear compartment adjacent to the Golgi complex (4), respectively. LIMPII and GLUT4 display negatively (Asp\(^{479}\)/Glut\(^{477}\)) and positively (Arg\(^{479}\)/Arg\(^{477}\)) charged residues respectively, at positions \(\pm 4\) and \(\pm 5\) upstream from the critical Leu residue. The change in the charge sign of residues \(\pm 4\) and \(\pm 5\) results in missorting of LIMPII and GLUT4. We note that the acidic Glu residue at position \(\pm 4\) is critical for efficient intracellular sorting of LIMPII to lysosomes, but is dispensable for its surface internalization by endocytosis. Efficient intracellular sorting and endocytosis of GLUT4 require an Arg pair between positions \(\pm 4\) and \(\pm 7\). These results are consistent with the existence of distinct leucine-based motifs and provide evidence of their different readings at different cellular sites.

Their structural determinants are, however, poorly characterized: a Leu paired with a second Leu, Ile, or Met is the only constant structural feature in these motifs, but the pair by itself lacks the ability to mediate transport (2, 8). Acidic residues located at positions \(\pm 4\) and \(\pm 5\) upstream from the Leu/Leu/Ile) pair have been demonstrated to be required for efficient internalization of invariant chain (Ii) and CD4 chimeras in mammalian cells (see Table I) (10, 11) and for transport of the t-SNARE Vam3 to yeast vacuoles (12). In addition, phosphorylation of a Ser residue at position 4 or 5 upstream is critical for the surface internalization of some membrane proteins (13–17). On the other hand, some of the Leu-based motifs lack upstream acidic residues in intracellular protein sorting and endocytosis, and this also strongly suggests the existence of a family of leucine-based motifs with distinct structural determinants and activities. The possibility of distinct specificity determinants that modulate the recognition of leucine-based motifs is also evident when the ability of specific clathrin adaptors to discriminate between leucine motifs is studied (11, 18–22).

Here we have studied the structural determinants involved in the Leu-mediated intracellular sorting and surface internalization of LIMPII and GLUT4, two membrane proteins with distinct cellular distributions. The results of our experiments indicate the existence of different Leu-based motifs endowed with distinct structural determinants. Furthermore, they show that Leu-based motifs use different structural determinants at different transport steps.

MATERIALS AND METHODS

Cell Culture—3T3-L1 fibroblasts were cultured for 48 h on plastic dishes or glass coverslips in normal medium (Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 4 mM glutamine, 50 mg/liter gentamycin, 100 mg/liter streptomycin, 100 IU/liter penicillin, and nonessential amino acids) in a humidified CO\(_2\) incubator at 37 °C. 3T3-L1 fibroblasts stably transfected with wild-type GLUT4 or GLUT4 mutants, when required, were converted into adipocyte-like (ADL) cells by incubation for 6–7 days in IDBX medium (normal medium supplemented with 10 μg/ml insulin, 10 μg/ml biotin, 0.25 μM dexamethasone, and 500 μM 1-isobutyl-3-methylxanthine). Clonal ADL cells displaying numerous lipid droplets of medium and large size in their cytoplasm were used before the onset of endogenous GLUT4 expression.

DNA Constructs—Wild-type rat GLUT4 and LIMPII cDNAs were cloned into the M13mp19 vector. All mutants were made by site-directed mutagenesis as described (23). For development of clonal cell lines of 3T3-L1 fibroblasts, constructs and mutants were cloned into a modified pPUR vector (CLONTECH) carrying the spleen focus-forming virus long terminal repeat promoter in the ApaI/EcoRI sites.

Transfection of Mammalian Cell Lines—To monitor the effects of the
developed by site-directed mutagenesis. The Asp 470/Glu471, Leu 475/
mutations on the distribution of LIMPII and GLUT4, COS-7 cells grown
to near confluency on glass coverslips for 48 h were transiently trans-
formed for 18 h only to prevent overexpression of the transfected pro-
teins and therefore saturation of the sorting mechanisms. Furthermore,
transfected cells were incubated for the last 3 h with 0.1 mM cyclohex-
imide to deplete the secretory pathway of transfected proteins. Mutants
displaying abnormal patterns of distribution were then studied in sta-
mables transfected 3T3-L1 fibroblasts. For stable protein expression,
clones were isolated within 2 weeks of transfection with the help of
Teflon rings and, following their expansion, were screened by immuno-
precipitation and Western blot analysis. pAb OSCR6 was able to immunoprecipitate detergent-solubilized
proteins. Clones were maintained thereafter with 5–7.5 mg/ml puromycin.

Antibodies—The rabbit polyclonal antibodies (pAb) OSCR6 (provided
by Dr. A. Zorzano, Universitat Barcelona) (26) and 828 were raised
against a peptide representing the entire cytoplasmic COOH tail of
LIMPII and GLUT4 as well as the mutated residues are
identifed by the one-letter code and are shown in position in the
diagrams of the LIMPII and GLUT4 COOH termini.

ADL cells washed twice with PBS and incubated for 3 min with cold (∼20 °C) methanol, washed three times, and incubated at 37 °C for 1 h with the corresponding specific antibody in PBS. After
being washed for 15 min with PBS, the cells were stained at 37 °C for 1 h with FITC-conjugated goat anti-mouse or goat anti-rabbit secondary
antibodies (Cappel, Durham, NC) prepared to 10 mg/ml in PBS con-
taining 0.25 mg/ml goat preimmune serum. Stained cells were washed
once more for 15 min with PBS, mounted on glass slides using Gelvatol
(Monsanto Co., St. Louis, MO), and studied under an Axiovert 135M
inverted microscope (Zeiss) or a confocal Radiance 2000 microscope
(Bio-Rad), and the fluorescence was photographed.

Quantitation of LIMPII and GLUT4 Cellular Levels—Stably trans-
fected 3T3-L1 fibroblasts grown to near confluency were washed with PBS
and incubated with 0.1 mM Na2CO3 (pH 11.3) at 4 °C for 30 min. Cell
extracts were centrifuged at 150,000 g for 30 min using a TL-100
ultracentrifuge (Beckman Instruments), and membrane pellets were
resolved by 10% SDS-PAGE. Resolved proteins were blotted onto nitro-
cellulose, immunoprobed with either mAb 29G10 or pAb OSCR6, and
studied using the ECL technique (Amersham Pharmacia Biotech, Ay-
lesbury, United Kingdom).

Subcellular Fractionation Studies—Low-density microsome (LDM)
and plasma membrane (PM)-enriched fractions were prepared from
stably transfected 3T3-L1 fibroblasts by a modification of the procedure
previously described (28). Briefly, the cells were washed with PBS,
scraped with a rubber policeman in 3 ml of cold HES buffer (20 mM

FIG. 1. LIMPII and GLUT4 mutants. The mutants listed were
developed by site-directed mutagenesis. The Asp470/Glu471, Leu475/
and Leu489/Ile490 pairs displayed in the COOH
termini of LIMPII and GLUT4 as well as the mutated residues are
identified by the one-letter code. Adip., adipocytes.

FIG. 2. Cellular levels of transfected LIMPII and GLUT4 proteins in stably transfected clonal 3T3-L1 fibroblasts. The levels of
wild-type and mutant LIMPII (A) and GLUT4 (B) were studied by
Western blot analysis using mAb 29G10 and pAb OSCR6. All lanes
were loaded with 50 μg of protein, except the LIMPII(Ala470/Ala471)
lane, which was loaded with 75 μg of protein. The major unspecific band
migrating ahead of GLUT4 is marked with an asterisk. Residues are
identified by the one-letter code. Adip., adipocytes.
HEPES (pH 7.4), 1 mM EDTA, and 255 mM sucrose), and disrupted by \( N_2 \) cavitation (2400 kilopascals). All manipulations were performed at 4 °C. Post-nuclear supernatants were fractionated by three consecutive centrifugations at 19,000 \( \times g \) for 20 min, then at 45,000 \( \times g \) for another 20 min, and finally at 180,000 \( \times g \) for 90 min to yield pellets enriched in LDMs. The membranes collected at 19,000 \( \times g \) were resuspended in HES buffer, layered onto a 35% sucrose cushion in 10 mM Tris (pH 7.4) and 1 mM EDTA, and recentrifuged at 108,000 \( \times g \) in an SW 40 rotor for 1 h. The white fluffy band recovered at the sucrose interface was then diluted 10-fold in 20 mM Tris and 2 mM EDTA, treated with 0.1 mM phenylmethylsulfonyl fluoride, and centrifuged at 150,000 \( \times g \) for 30 min to yield a pellet enriched in plasma membranes. LDM and PM pellets were mixed for 1 h at 4 °C with 300 \( \mu \)l of extraction buffer, and the insoluble material was removed by centrifugation at 150,000 \( \times g \) for 30 min. The resulting supernatants were scrutinized for GLUT4 by Western blot analysis as described above.

**Glucose Uptake Studies**—Untransfected and stably transfected 3T3-L1 fibroblasts cultured on 10-mm coverslips were quickly washed.
with Krebs-Ringer HEPES buffer (KRHB) and incubated at 37 °C for 5 or 10 min with 15 ml of KRHB containing 0.2% bovine serum albumin and 1 mM 2-deoxy[3H]glucose (specific activity = 6.66 Ci/mmol; PerkinElmer Life Sciences). Nonspecific 2-deoxyglucose uptake was measured in cells preincubated for 5 min with 15 ml of KRHB containing 50 μM cytochalasin B and 40 mM glucose, and the uptake assay was performed in the presence of both cytochalasin B and glucose. Glucose uptake was stopped by washing the cells quickly three times with 1 ml of ice-cold PBS containing 50 mM glucose. Cells were lysed with 200 μl of 0.1 N NaOH to measure protein, and radioactivity was estimated by scintillation counting. 2-Deoxyglucose uptake was normalized to cpm/μg of protein/min.

Studies of LIMPII and GLUT4 Intracellular Sorting—Clonal 3T3-L1 fibroblasts cultured for 48 h to 80% confluency on 10-cm dishes were washed twice with methionine/cysteine-free Dulbecco’s modified Eagle’s medium and metabolically labeled for 30 min with 0.25 mCi/ml [35S]methionine/cysteine (specific activity > 1000 Ci/mmol; PRO-MIX, Amersham Pharmacia Biotech) prepared in Dulbecco’s modified Eagle’s medium and 2% dialyzed fetal calf serum. The 30-min pulse was adequate to metabolically label LIMPII and GLUT4 and also to begin to monitor their appearance at the plasma membrane since they required 45 and 20 min, respectively, to traverse the Golgi (27, 29). Cells were chilled on ice at different times after labeling, and surface-exposed LIMPII and GLUT4 were biotinylated using sulfo-NHS-LC-biotin (Pierce) and Bio-LC-ATB-BMPA, 9 and 45 and 20 min, respectively, to traverse the Golgi (27, 29). Cells were metabolically labeled as described above and then labeled for 1 min at 4 °C with 1 mM Bio-LC-ATB-BMPA prepared in 250 ml of KRHB (31). Biotinylated samples were then washed and chased for different time periods at 37 °C in KRHB. Quantitation of biotin–35S-labeled GLUT4 retained on the cell surface was carried out using purified plasma membrane fractions (see “Subcellular Fractionation Studies”).

Studies of Transport of Newly Synthesized LIMPII Molecules to Lysosomes—Cells metabolically labeled for 15 min as described above were disrupted by N2 cavitation, and the post-nuclear supernatant was mixed as described to give a final concentration of 20% Percoll (22). Fractions of 3.3 ml made to 1% Triton X-100 were incubated at 4 °C for 30 min, and insoluble material and Percoll were removed by centrifugation. LIMPII was immunoprecipitated by incubation at 4 °C for 2 h with 10 μl of mAb 29G10/protein G-Sepharose and quantitated by scanning the autoradiograms produced by SDS-PAGE.

RESULTS

The only constant determinant in the leucine-based motifs is a Leu pair that is followed by a COOH residue with a long aliphatic chain (Ile, Met, and Val). In addition, negatively charged residues at position 4 upstream have been implicated as being involved in membrane protein endocytosis in mammalian cells as well as in the targeting of Vamp3 to yeast vacuoles (10–12).

Sorting of LIMPII and GLUT4, two membrane proteins with distinct cellular distributions, is mediated by leucine-based motifs, as shown by the accumulation of mutants with ablated leucine motifs in the plasma membrane (1, 2, 4). Interestingly, whereas in LIMPII, the residues at positions 4 and 5 upstream are negatively charged (Asp470/Glu471), in GLUT4, they are
positively charged (Arg484/Arg485). To further characterize the structural determinants contained in the leucine-based motifs, the Asp470/Glu471 and Arg484/Arg485 pairs were (a) substituted by pairs of opposite charge, (b) replaced by two uncharged residues, (c) split, and (d) separated from the downstream critical Leu residue (Fig. 1). The effect of these modifications on the cellular distribution, intracellular sorting, and surface internalization of LIMPII and GLUT4 was studied by microscopy and biochemical means.

To exclude any side effects of overexpression on the distribution of the transfected proteins, the clones selected by microscopy were studied for their levels in the transfected proteins by Western blot analysis. The selected clones expressed comparable or slightly lower levels of the LIMPII and GLUT4 mutants as compared with the wild-type proteins (Fig. 2, A and B). Furthermore, the cellular levels of the GLUT4 mutants were comparable to those of endogenous GLUT4 in 3T3-L1 adipocytes and rat adipocytes (Fig. 2B).

**Leu-based Signals in LIMPII and GLUT4 Comprise Different Upstream Structural Motifs**—To study whether the Asp470/Glu471 and Arg484/Arg485 pairs could modify function indistinctly in the context of LIMPII and GLUT4, these residues were replaced by pairs of opposite charge (two Arg and two Glu residues, respectively), and the cellular distributions of the resulting mutants and the native proteins were compared by microscopy.

In clonal 3T3-L1 fibroblasts, the bulk of wild-type LIMPII was localized to lysosomes (Fig. 3), whereas GLUT4 was found in a reticular perinuclear compartment (GLUT4 storage compartment) and in vesicles scattered throughout the cytoplasm (Fig. 4), as shown by immunofluorescence microscopy. In 3T3-L1 fibroblasts as well as in other cell lines studied (COS, normal rat kidney, baby hamster kidney, and Chinese hamster ovary), anti-LIMPII antibodies did not stain the plasma membrane, even under conditions of protein overexpression, whereas anti-GLUT4 antibodies stained it weakly in a small number of cells.

Introduction of the Arg470/Arg471 and Glu484/Glu485 mutations in LIMPII and GLUT4, respectively, provoked a dramatic accumulation of both proteins at the plasma membrane (Figs. 3 and 4), indicating a strong inhibition of their normal sorting. Because phosphorylated residues upstream of leucine motifs have been shown to be critical for protein endocytosis (13–17) and Thr488 was lost during the development of the LIMPII(Arg470/Arg471) mutant, we checked whether the accumulation of LIMPII(Arg470/Arg471) in the plasma membrane was pro-
voked by the loss of Thr\textsuperscript{468}. The answer was negative; a study of the distribution of the LIMPII(Ala\textsuperscript{468}) mutant showed that the replacement of Thr\textsuperscript{468} by Ala did not provoke the accumulation of the protein in the plasma membrane (data not shown), therefore eliminating that possibility.

The effect of replacing the Asp\textsuperscript{470}/Glu\textsuperscript{471} pair by two uncharged residues on the distribution of LIMPII was also studied. The study, first performed in transiently transfected COS-7 cells, revealed a weak but significant staining of their plasma membrane (data not shown), suggesting a limited accumulation of the LIMPII(Ala\textsuperscript{470}/Ala\textsuperscript{471}) mutant in that location. When the same experiment was repeated in stably transfected 3T3-L1 fibroblasts, the weak staining of the plasma membrane was again observed (Fig. 3; see also Fig. 6 below), thus confirming that the replacement of the Asp\textsuperscript{470}/Glu\textsuperscript{471} pair by two uncharged residues affected the trafficking and distribution of LIMPII. Altogether, these observations indicate the importance of the –4 and –5 charged residues in the Leu-mediated sorting of LIMPII and GLUT4 and show that the effect of charge sign on their distribution is conditioned by the protein context in which the Leu-based motif is expressed.

The Asp\textsuperscript{470}/Glu\textsuperscript{471} and Arg\textsuperscript{484}/Arg\textsuperscript{485} pairs were further manipulated by (a) inserting an Ala between the two paired residues and (b) by increasing their distance to the critical leucine. Splitting of the Asp\textsuperscript{470}/Glu\textsuperscript{471} pair in LIMPII, produced by replacing Ala\textsuperscript{469} and Asp\textsuperscript{470} by Asp and Ala, respectively (Fig. 1), did not affect the distribution of the protein, which was confined within lysosomes in transiently transfected COS-7 cells immunostained with mAb 29G10 (data not shown). An acidic residue at position –5 was therefore not critical for LIMPII sorting. In sharp contrast, the splitting of the Arg\textsuperscript{484}/Arg\textsuperscript{485} pair in GLUT4, produced by replacing Phe\textsuperscript{483} and Asp\textsuperscript{484} by Arg and Ala, respectively (Fig. 1), provoked the deflection of GLUT4 to the plasma membrane both in COS-7 cells (data not shown) and in 3T3-L1 fibroblasts stably expressing GLUT4(Arg\textsuperscript{483}/Arg\textsuperscript{485}) immuno-stained with pAb 828 (Fig. 4). We note that this effect was not produced by the replacement of Phe\textsuperscript{483} by Arg since the distributions of the GLUT4(Ala\textsuperscript{483}) mutant and GLUT4 in transiently transfected COS-7 cells were identical (data not shown).

The differences between the Asp\textsuperscript{470}/Glu\textsuperscript{471} and Arg\textsuperscript{484}/Arg\textsuperscript{485} determinants were further explored by studies of the distribution of LIMPII(Glu\textsuperscript{471}/Leu\textsuperscript{477}) and GLUT4(Asp\textsuperscript{470}/Leu\textsuperscript{491}) mutants in which the distance between Asp\textsuperscript{470}/Glu\textsuperscript{471}/Arg\textsuperscript{484}/Arg\textsuperscript{485} and the critical leucine was increased by the insertion of two Ala residues (Fig. 1). The results of these studies showed that moving the Asp\textsuperscript{470}/Glu\textsuperscript{471} pair away strongly affected the correct targeting of LIMPII to lysosomes (Fig. 3), as shown by its dramatic accumulation in the plasma membrane, whereas moving the Arg\textsuperscript{484}/Arg\textsuperscript{485} pair did not alter the distribution of GLUT4 (Fig. 4).

When the GLUT4 distribution studies were repeated in 3T3-L1 fibroblasts that were treated for 6–8 days with IDBx medium (see “Materials and Methods”) and that developed large lipid droplets in their cytoplasm, but did not express endogenous GLUT4, the results (Fig. 5) were comparable to those with undifferentiated fibroblasts. To quantitate the accumulation of the LIMPII mutants in the plasma membrane of 3T3-L1 fibroblasts, cells stably expressing wild-type LIMPII or its mutants were fixed at 4 °C for 1 h with 2% paraformaldehyde, permeabilized or not with 1% Triton X-100, and incubated with mAb 29G10 for 1 h at 4 °C, and then their fluorescence was quantitated by flow cytometry. The results of these studies showed that 1.5% of wild-type LIMPII, 3% of LIMPII(Ala\textsuperscript{470}/Ala\textsuperscript{471}), and >45% of LIMPII(Asp\textsuperscript{470}/Arg\textsuperscript{471}) and LIMPII(Glu\textsuperscript{471}/Leu\textsuperscript{477}) were exposed at the plasma membrane (Fig. 6).

Attempts to quantitate the surface levels of GLUT4 in stably transfected 3T3-L1 fibroblasts by flow cytometry failed due to the lack of reactivity of a pAb antibody raised against the large exofacial loop of GLUT4 unless this was interrupted by the introduction of the hemagglutinin tag.\textsuperscript{2} The surface levels of GLUT4 were therefore estimated (a) by quantification of its levels in subcellular fractions enriched in PMs and LDMs and (b) by measuring the glucose uptake by the cells (Fig. 7).

The steady-state levels of wild-type GLUT4 and GLUT4 mutants in PMs and LDMs were measured by Western blot analysis using pAb OSCR6 and the ECL technique. The results showed that the amounts of wild-type GLUT4 and GLUT4(Arg\textsuperscript{485}/Leu\textsuperscript{491}) recovered with the plasma membrane were only 5 and 6% of the amounts recovered with the LDM fraction, whereas 20% of GLUT4(Asp\textsuperscript{484}/Glu\textsuperscript{485}) and 45% of GLUT4(Arg\textsuperscript{485}/Arg\textsuperscript{485}) were recovered with the PM fraction (Fig. 7A).

These results were confirmed by glucose uptake studies performed in clonal 3T3-L1 fibroblasts and ADL cells. It is impor-
tant to note that all the clones studied expressed comparable levels of ubiquitous GLUT1 (Fig. 7B). We observed that 3T3-L1 fibroblasts stably expressing GLUT4(Glu 484/Glu485) and GLUT4(Arg483/Arg485) transported nearly 3-fold more glucose than fibroblasts expressing wild-type GLUT4 or GLUT4(Arg485/Leu491) (Fig. 7C). Furthermore, the same differences were observed when the glucose uptake was studied in ADL cells (Fig. 7D).

Altogether, these observations confirmed the results of the immunofluorescence microscopy studies. They showed that an acidic residue at position 4 upstream from Leu475 was critical for correct distribution of LIMPII. By contrast, correct distribution of GLUT4 required a pair of upstream basic residues whose distance to Leu489 was not so critical. These results indicated the existence of important structural differences between the leucine-based motifs in the LIMPII and GLUT4 molecules.

**Fig. 7.** Surface levels of wild-type GLUT4 and mutants with manipulated Arg484 and Arg485 residues. A, clonal 3T3-L1 fibroblasts stably expressing either wild-type GLUT4 (wt-GLUT4) or the indicated mutants were lysed and fractionated, and the transfected proteins recovered with the plasma membrane were quantitated by Western blot analysis using pAb OSCR6. The experiment shown is representative of four independent experiments. B, plasma membrane fractions purified from 3T3-L1 fibroblasts and the ADL cells stably transfected with the indicated GLUT4 proteins were scrutinized for their content of GLUT1 by Western blot analysis. C and D, glucose uptake in 3T3-L1 fibroblasts and ADL cells, respectively, stably expressing the indicated GLUT4 proteins was measured as described under “Materials and Methods.” Results are expressed as a proportion of the PM levels or the glucose uptake by clonal cells stably expressing wild-type GLUT4. Plotted values are the mean of two independent experiments and duplicate samples.

**Mutations of Residues at Positions 4 and 5 Upstream from Leu475 and Ile476 Reveal Differences in the Structural Requirements for Efficient Intracellular Sorting and Surface Internalization of LIMPII**—To investigate the role of the Asp470/Glu471 pair in the intracellular sorting of LIMPII, we studied the effects of mutations on the rate of appearance of newly synthesized molecules on the cell surface. To monitor this appearance, clonal 3T3-L1 fibroblasts stably expressing wild-type LIMPII and the LIMPII(Arg470/Arg471), LIMPII(Ala470/Ala471), and LIMPII(Glu471/Leu477) mutants were metabolically labeled for 30 min with [35S]methionine/cysteine, and the molecules remaining on the plasma membrane at various chase times (20 min, 40 min, and 3 h) were biotinylated with the membrane-impermeable reagent sulfo-NHS-LC-biotin and precipitated with streptavidin-Sepharose and then with mAb 29G10. The analysis of these precipitates by autoradiography after 10% SDS-PAGE showed that the amounts of LIMPII(Arg470/Arg471), LIMPII(Ala470/Ala471), and LIMPII(Glu471/Leu477) recovered after a 20-min chase were 3–4-fold higher than the amount of wild-type LIMPII recovered (Fig. 8). This difference strongly suggested that the intracellular sorting of these three mutants was inhibited. Interestingly, a time course study revealed that although the amount of newly synthesized LIMPII(Ala470/Ala471) recovered from the cell surface decreased with longer chase periods, the amounts of LIMPII(Arg470/Arg471) and LIMPII(Glu471/Leu477) were either retained at the cell surface or return there after internalization (Fig. 8). These observations agreed with the results of immunofluorescence microscopy studies showing that the plasma membrane of fibroblasts expressing LIMPII(Arg470/Arg471) and LIMPII(Glu471/Leu477) was stained more strongly than that of fibroblasts expressing LIMPII(Ala470/Ala471). Together with the confinement of wild-type LIMPII in lysosomes, the results indicated that the acidic residue at position 4 upstream from Leu475 was critical for efficient intracellular sorting of LIMPII.

The rates of internalization of wild-type LIMPII and the three mutants, produced by manipulation of the Asp470/Glu471 pair, were compared using a flow cytometry technique. Stably transfected 3T3-L1 fibroblasts were incubated for 1 h at 4 °C with mAb 29G10, and after washing and incubation at 37 °C for different time periods, the protein remaining at the cell surface was stained with an FITC-conjugated goat anti-mouse secondary pAb. From measurements of the fluorescence remaining at the cell surface by flow cytometry, we observed that wild-type LIMPII and LIMPII(Ala470/Ala471) were rapidly internalized, whereas LIMPII(Arg470/Arg471) and LIMPII(Glu471/
LIMPII and LIMPII(Ala470/Ala471) recovered with lysosomes after 2 h of chase, when the percentages of wild-type with lysosomes (Fig. 10). These differences were roughly maintained differences in the role of Glu471 in the intracellular sorting of LIMPII(Ala470/Ala471) and none of LIMPII(Arg470/Arg471) were recovered for 15 min with [%35S]methionine/cysteine and then chased for 45 min or 2 h with normal medium. The Golgi and plasma membrane was studied in clonal 3T3-L1 fibroblasts pulse-labeled with [%35S]methionine/cysteine and a 40-min chase period, we found that the fraction of newly synthesized wild-type GLUT4 detected at the cell surface was 1.7%, whereas the fractions of GLUT4(Glu484/Leu485) and GLUT4(Arg483/Arg485) were 3.5 and 5.1%, respectively (Fig. 11). The comparatively faster appearance of GLUT4(Glu484/Leu485) and GLUT4(Arg483/Arg485) on the plasma membrane strongly suggested that their intracellular sorting was inhibited.

To study the effects of the Arg483/Arg485 manipulation on the surface internalization of GLUT4, 3T3-L1 fibroblasts stably transfected with wild-type GLUT4, GLUT4(Glu484/Leu485), and GLUT4(Arg483/Arg485) were surface-biotinylated at 4 °C for 1 min with 1 mM Bio-LC-ATB-BMPA. Cells were then washed and incubated at 37 °C for 10, 20, or 40 min in normal medium, and then the membranes were fractionated. The biotinylated proteins were extracted from the PM-enriched fraction and precipitated with streptavidin-Sepharose, and GLUT4 was quantitated by Western blot analysis using pAb OSCR6. We observed that the effects of the Arg483/Arg485 manipulation on the internalization of GLUT4 were similar to those on its intracellular sorting: GLUT4 was internalized faster than GLUT4(Glu484/Leu485), which was internalized faster than GLUT4(Arg483/Arg485) (Fig. 12).

**DISCUSSION**

Leucine-based transport motifs displayed in the cytoplasmic tails of membrane proteins have been implicated in their sorting at the TGN and endosomes and surface internalization by endocytosis (for reviews, see Refs. 8, 33, and 34). The reading of leucine-based motifs at different cellular sites and the distinct cellular distribution of the clathrin adaptors involved in their recognition (for reviews, see Refs. 35–37) strongly suggest the existence of related leucine-based motifs with different transport specificities. This possibility is also suggested by the fact that pairing of a Leu residue to a second Leu, Ile, Met, or Val (8), while critical, is insufficient for transport. In addition, the upstream acidic residues described in some dileucine motifs (Table 1) (10–12) are absent in others.

To investigate the possible existence of different leucine-based motifs, we first compared the sequences adjacent to the dileptide in LIMPII and GLUT4, two membrane proteins sorted by leucine-based motifs to lysosomes and to a tubulovesicular storage compartment adjacent to the Golgi complex, respectively (1–3, 38–40). The comparison of their dileucine motifs reveals that the pair of acidic residues at positions −4 and −5 in LIMPII, and shared by other membrane proteins with leucine-based motifs (see Table I), is replaced in GLUT4 by a pair of Arg residues or by the pair His/Arg (41). It should be pointed that although the presence of upstream Arg residues were 30 and 24%, respectively, as compared with 5–10% of LIMPII(Arg470/Arg471) and LIMPIII(Glu472/Leu477) (Fig. 10). These results showed that the LIMPII molecules deflected to the plasma membrane reached the lysosomes at rates reflecting their rates of internalization from the plasma membrane.
is not uncommon in leucine-based motifs (see Table I), there have been no studies regarding their role in transport. The accumulation of LIMPII and GLUT4 at the plasma membrane after the exchange of their pairs of acidic and basic residues shows that these structural determinants built in their leucine-based motifs are recognized as being different. The distinct effects of their manipulation indicates that the character and position of the Asp470/Glu471 and Arg484/Arg485 pairs have distinct influence on the distribution of the two proteins. For example, the accumulation of LIMPII at the plasma membrane upon the insertion of two alanines between the Asp470/Glu471 pair and Leu475 and the lack of effect on GLUT4 distribution when they were inserted between Arg484/Arg485 and Leu489 indicate that an acidic residue at position 471 is critical for efficient targeting of LIMPII to lysosomes, whereas the basic Arg residue can be moved away from this position in the GLUT4 molecule without affecting its targeting. Moreover, the pairing of the two Arg residues is required for efficient sorting of GLUT4 to the storage compartment adjacent to the Golgi complex, as shown by its accumulation at the

![Fig. 9.](image1.png)

**Fig. 9.** Surface internalization of wild-type LIMPII and mutants with manipulated Asp^470^ and Glu^471^ residues. Stably transfected clonal 3T3-L1 fibroblasts expressing wild-type LIMPII or the mutants indicated were incubated at 4 °C for 1 h with mAb 29G10. After washing free of excess antibody, the cells were surface-labeled with an FITC-conjugated goat anti-mouse pAb and then incubated at 37 °C for the indicated times. The fluorescence remaining at the cell surface was quantitated by flow cytometry, and the log of its intensity (abscissa), expressed in arbitrary units, is plotted against the relative cell number (ordinate). Numbers indicate the percentage of LIMPII remaining at the plasma membrane. The contrast between the rapid internalization of wild-type LIMPII and LIMPII(Ala^470^/Ala^471^) as compared with the internalization of LIMPII(Arg^470^/Arg^471^) and LIMPII(Glu^471^/Leu^477^). The experiment shown is representative of two independent experiments.

![Fig. 10.](image2.png)

**Fig. 10.** Transport to lysosomes of newly synthesized wild-type LIMPII and mutants with manipulated Asp^470^ and Glu^471^ residues. Clonal 3T3-L1 fibroblasts stably expressing either wild-type LIMPII (wt-LIMPII) or the indicated mutants were pulse-labeled for 15 min with [35S]methionine/cysteine and chased for 45 min or 2 h at 37 °C with normal medium. At each chase time point, the cells were disrupted by N₂ cavitation, and the post-nuclear supernatants were fractionated by centrifugation on 20% Percoll gradients as described (28). The 10-ml gradients were divided into three fractions, and LIMPII was immunoprecipitated with mAb 29G10, resolved by SDS-PAGE, and studied by fluorography. Fractions 1 and 3 were enriched in lysosomes and plasma membrane, respectively. The experiment shown in A is representative of three separate experiments. B shows the distribution of LIMPII between fractions 1 (open bars) and 3 (hatched bars) calculated after scanning the films shown in A.
plasma membrane following its splitting by the insertion of one Ala residue, whereas the pairing of the Asp^{470} and Glu^{471} residues in the LIMPII molecule appears to be less critical for targeting of the LIMPII(lAsp^{470}/Glu^{471}) mutant to lysosomes. It is important to note that although acidic pairs are often found, the protein bands shown in Fig. 5 were extracted using streptavidin-Sepharose. Precipitated GLUT4 was then quantified by Western blot analysis using pAb OSCR6. The relative decreases were measured by scanning the Western blots and are indicated (Percentage). The experiment shown is representative of two separate experiments.

To study the effect of manipulating the upstream determinants on the intracellular sorting of LIMPII and GLUT4, probably at or near the TGN, we have compared the rate of appearance at the cell surface of newly synthesized molecules of the wild-type and mutant proteins. The faster appearance of all the LIMPII and GLUT4 mutants that accumulate at the plasma membrane is a strong indication of their inefficient intracellular sorting. Furthermore, the deflection to the cell surface of mutants developed by substituting the pairs of acidic and basic residues by pairs of opposite charge indicates the different structure of the two leucine motifs studied here. This observation suggests that though the intracellular sorting of LIMPII and GLUT4 is catalyzed by leucine motifs, the nature of the transport steps and that of the machineries involved in their recognition are probably different. The recent observations that AP-3 binds to a synthetic peptide from the COOH terminus of LIMPII, but not to GLUT4 (22), and that GLUT4 and CD3γ synthetic peptides bearing the leucine-based sorting motif compete for binding to AP-1 (19) suggest their recognition by different clathrin adaptors. However, very little is known about the organization of TGN subcompartments or the sorting machineries operating there. It seems unlikely that the sorting of the two proteins studied here occurs in the same TGN subcompartment. The localization of AP-1 and AP-3 to what appears to be distinct compartments within the TGN area (47, 48) suggests that the Leu-based sorting of LIMPII and GLUT4 could occur in different subcompartments. Further analysis of TGN subcompartments may show where and how LIMPII and GLUT4 are sorted after traversing the Golgi cisternae. The distinct sorting of LIMPII and GLUT4 is less striking when compared with soluble and membrane lysosomal proteins, which are sorted by Leu-based mechanisms into different clathrin adaptors. However, very little is known about the organization of TGN subcompartments or the sorting machineries involved in their recognition are probably different. The recent observations that AP-3 binds to a synthetic peptide from the COOH terminus of LIMPII, but not to GLUT4 (22), and that GLUT4 and CD3γ synthetic peptides bearing the leucine-based sorting motif compete for binding to AP-1 (19) suggest their recognition by different clathrin adaptors. However, very little is known about the organization of TGN subcompartments or the sorting machineries operating there. It seems unlikely that the sorting of the two proteins studied here occurs in the same TGN subcompartment. The localization of AP-1 and AP-3 to what appears to be distinct compartments within the TGN area (47, 48) suggests that the Leu-based sorting of LIMPII and GLUT4 could occur in different subcompartments. Further analysis of TGN subcompartments may show where and how LIMPII and GLUT4 are sorted after traversing the Golgi cisternae. The distinct sorting of LIMPII and GLUT4 is less striking when compared with soluble and membrane lysosomal proteins, which are sorted by Leu-based mechanisms into different clathrin coated vesicles and yet have the same subcellular destiny (49, 50).

The sorting of LIMPII(lAsp^{470}/lAla^{471}) is of great interest. Although the replacement of the acidic residues at positions −4 and −5 by two uncharged alamines strongly inhibited the intracellular sorting of LIMPII, it did not affect its internalization, and as a result, its level of accumulation at the cell surface was relatively small. These observations clearly show that the requirements for the Glu residue at position −4 in the intra-
In part A, leucine-based motifs involved in intracellular sorting and/or cell-surface endocytosis that present upstream acidic residues are shown. The critical leu Leu(470/471) pair is boxed and shown in boldface italic letters. Sequences were taken from the indicated references: LIMPII (2, 3), Lip31 (61), CI-M6PR (7), CD-M6PR (6), CD3 (95), sortilin (62), Vamp3 (12), tyrosinase-related protein-1 (TRP-1; gp75) (63), tyrosinase (64), alkaline phosphatase (ALP) (65), Pml-17 (melanosomal membrane protein) (66), P-protein (putative tyrosine transporter in melanosomal membranes) (63), VMAT2 (neuron-specific vesicular monoamine transporter) and VACHT (vesicular acetylcholine transporter) (12), TrkA (I. V. Sandoval, S. Martinez-Arca, J. Valdueza, Silvia Palacios, and G. D. Holman, unpublished results), CD44 (plasma membrane adhesion protein), (68), V2 receptor (vasopressin receptor) (69), insulin receptor (IR) (70), and human immunodeficiency virus-1 (HIV-1) Nef (71). In part B, leucine-based motifs with N-terminal arginine residues are shown. In addition to the critical hydrophobic pair, boxed and in boldface, Arg residues upstream and near the Leu/Leu (Leu) pair are indicated in boldface italic letters and boxed. Sequences were taken from the indicated references: GLUT4 (1), insulin-regulated aminopeptidase (IRAP) (56), CD-M6PR (6), α2A-adrenergic receptor (α2AR), α2B-adrenergic receptor (α2BR), and α2C-adrenergic receptor (α2CR) (69); Gap1p (general amino acid permease) (72).

### Table I

| Protein Trafficking Mediated by Leucine-based Motifs |
|-----------------------------------------------------|
| Leucine-based motifs containing N-terminal acidic and basic residues |
| LIMPII | A | D | E | R | A | P | L | R |
| ii | M | D | D | Q | R | D | S |
| CD-M6PR | D | D | S | D | D | E | D | L |
| CD3 | A | D | D | R | V | G | L |
| CD35 | E | E | R | D | D | H | H |
| Sortilin | D | D | S | D | D | E | D | L |
| Vamp3 | V | N | E | Q | S | P |
| Tyrosinase | K | D | D | Y | H | S | Y |
| ALP | P | S | E | Q | T | R | V | P |
| CD4 | L | G | E | N | S | P |
| P-protein | F | K | E | D | T | P | W |
| VMAT2 | K | E | E | K | M |
| VACHT | R | S | E | R | D | V |
| MNK | F | P | D | K | H | S | L | V |
| V2 | S | S | E | L | R | S |
| IR | S | R | E | K | Y | T | L | E |
| HIV-1 Nef | T | G | E | N | S | L | L |

Regarding the motifs involved in the sorting of membrane proteins at the TGN and plasma membrane, it is noteworthy that in addition to the leucine motifs, the tyrosine-based motifs Y(F)/(X)Z are also implicated in these two transport steps (Refs. 57 and 58; for reviews, see Refs. 8, 9, and 59). In fact, evidence showing the influence of the motif position relative to the membrane and the effect of the residues surrounding the critical tyrosine suggests the existence of a family of tyrosine-based motifs (30, 60). The fact that membrane proteins display leucine and/or tyrosine-based motifs, in one or multiple copies (8), strongly suggests that all combinations endow proteins with the unique trafficking capacity required for their specialized normal cellular distributions.

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Distinct Reading of Different Structural Determinants Modulates the Dileucine-mediated Transport Steps of the Lysosomal Membrane Protein LIMPII and the Insulin-sensitive Glucose Transporter GLUT4

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