The Carboxyl Terminus of GLUT4 Contains a Serine-Leucine-Leucine Sequence That Functions as a Potent Internalization Motif in Chinese Hamster Ovary Cells*

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To characterize the trafficking motifs contained in the carboxyl terminus of GLUT4, a chimera (GTCTR) was constructed in which the carboxyl-terminal 30 amino acids of GLUT4 were substituted for the amino-terminal cytoplasmic domain of the transferrin receptor (TR). The endocytic behavior of this chimera was characterized in Chinese hamster ovary cells. The GTCTR chimera had a more predominant intracellular distribution compared to the TR. Only 20% of the GTCTR chimera is on the surface at steady-state compared to 35% of the TR. The GTCTR chimera is internalized 50% more rapidly and recycled 20% more slowly than the TR. Acidification of the cytosol inhibited internalization of the GTCTR chimera, indicating that the chimera is internalized through clathrin-coated pits. Mutations of GTCTR were constructed in which a di-leucine sequence of the carboxyl domain of GLUT4 was mutated to a di-alanine sequence (GTCTR-AA) and serine residue 488, immediately preceding the di-leucine sequence, was mutated to either an alanine or aspartate residue. In each case, albeit to varying degrees, the substitutions shifted the distribution of the mutated GTCTR constructs toward the surface. The shift in the distribution of GTCTR-AA resulted from a 10-fold reduction in internalization, and the shift of serine 488 mutants resulted from a 3-fold reduction in the internalization rate compared to GTCTR. None of these mutations affected the recycling rate. These results demonstrate that the carboxyl terminus of GLUT4 contains a serine-leucine-leucine-based motif that, when expressed in non-insulin responsive cells, functions as a potent internalization motif which promotes more rapid internalization than does the native TR internalization motif.

In appropriate target tissues insulin stimulates a 10–30-fold increase in glucose uptake by recruiting the glucose transporter isoform, GLUT4, from intracellular compartments to the surface (1–4). In the basal-state, ≈10% of GLUT4 is on the cell surface, and following insulin treatment, ≈50% of GLUT4 is on the surface (4, 5). In both the basal and insulin-treated states GLUT4 is constitutively internalized and recycled, and therefore the fraction of GLUT4 on the surface is determined by the rates of GLUT4 internalization and recycling (6, 7). Many cell surface membrane proteins are constitutively internalized and recycled. In general, 30–50% of constitutively cycling membrane proteins are on the surface at steady-state. The more pronounced intracellular accumulation of GLUT4 in the basal-state is principally determined by the slow rate of GLUT4 recycling. In fat cells GLUT4 is recycled back to the cell surface at one-fifth to one-tenth the rate of other recycled proteins (6–8). Most membrane proteins are recycled by a default, bulk flow process (9). Consequently, GLUT4 must contain structural information that slows its recycling in the basal state. The insulin-induced increase in GLUT4 on the surface is achieved by a 300–900% increase in the rate of GLUT4 recycling and a 30–70% decrease in the rate of internalization, with both changes contributing to the increase in cell surface GLUT4 (6, 7, 10).

GLUT1, another glucose transporter isoform, is evenly distributed between the interior and the surface of cells in the basal-state (7). To identify the domains of GLUT4 that direct its isotype-specific endocytic trafficking, chimeras between GLUT4 and GLUT1 have been studied. In one set of studies, a GLUT4-like distribution was conferred to GLUT1/GLUT4 chimeras by the cytoplasmic amino terminus of GLUT4 (11, 12). Specifically, a FQQI motif within the amino cytoplasmic domain of GLUT4 was identified as the motif that determines the distribution of GLUT4 (12). Other studies identified the cytoplasmic carboxyl-terminal 30 amino acids of GLUT4 as the region most important in determining the distribution of GLUT4 (13–15). A di-leucine sequence in the carboxyl terminus of GLUT4 was shown to be required for the intracellular concentration of the transporter. Mutation of these leucines to alanines shifted the distribution to the surface (16, 17). It has also been reported that GLUT4 sequences outside the amino and carboxyl cytoplasmic domains are involved in determining GLUT4's distribution (18). In these previous studies the rates of internalization and recycling were not measured. Consequently, the changes in the kinetic parameters that determine the distribution of the constructs were not identified.

Transferrin receptor (TR) is a constitutively recycled membrane protein whose ligand, transferrin (Tf), mediates cellular uptake of iron (19). The Tf-TR complex is internalized through clathrin-coated pits into acidic endosomes where dfferic Tf releases iron. Iron-free Tf remains associated with the TR and is returned to the cell surface. At the neutral extracellular pH, iron-free Tf is released from the TR (19). A number of biochemical and microscopy assays have been developed that allow for the detailed analysis of each step of TR internalization and recycling.

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1 The abbreviations used are: TR, transferrin receptor; Tf, transferrin; CHO, Chinese hamster ovary; GTCTR, cytoplasmic domain of the TR; PCR, polymerase chain reaction; MES, 4-morpholineethanesulfonic acid; wt, wild-type.
GLUT4 C-terminal SLL Internalization Motif

Aspartate codon of GLUT4 fused in-frame to the codons for residue 60 of the TR. PCR fragment C, containing sequences coding for residues 505 to 509 of GLUT4 fused in-frame to sequences coding for residues 60 to 64 of the TR was synthesized using an oligonucleotide in the carboxy-terminal 30 amino acids of GLUT4 to the complete transmembrane and partial ecto-domain of the TR. Fragment C was joined with the rest of the TR sequences as described previously (22). This ligation joined the chimeric portion of the construct to the remainder of the TR (F11). The GTCTR-A chimera, the GTCTR-S488D chimera, and the GTCTR-S488D chimera were constructed by oligonucleotide-directed mutagenesis of the GTCTR cDNA. The cytoplasmic and transmembrane domains of the PCR generated constructs were sequenced by the Columbia University Cancer Center (ABI sequencing).

TR Trafficking Assays—The kinetic assays used to characterize the rates of internalization, recycling, and steady-state surface-to-internal distribution, and iron accumulation have been described elsewhere (21, 22). For each assay, cells were grown in six-well clusters to a density of 5 x 10⁶ cells/35-mm well. Two wells per plate were used to determine nonspecific binding of TF by including a 100-fold excess of unlabeled TF incubated in the incubation medium. All data have been corrected for nonspecific binding, which was typically less than 10% of the total. The binding buffer used for experimental incubations was McCoy's 5A medium supplemented with 50 mM sodium bicarbonate, 220 mM sodium bicarbonate, 20 mM HEPES, pH 7.4 (medium 1). The neutral wash buffer was 150 mM NaCl, 20 mM HEPES, 1 mM CaCl₂, 5 mM KCl, 1 mM MgCl₂, pH 7.4 (medium 2).

Steady-state Distribution—The amount of chimeras on the surface was determined by measuring the amount of TF binding in medium 1 at 4°C. Cells were preincubated in medium 1 for 1 h at 37°C in 5% CO₂ and then incubated with 3 μg/ml iodinated TF in medium 2 on ice for 2 h. The cells were washed with 4°C medium 2, solubilized in 0.1% Triton X-100, 0.1 M NaOH, and the radioactivity determined by γ-counting. At 4°C endocytic trafficking is blocked so that the amount of cell-associ- ated TF reflects the amount of TF bound to the surface of cells. The internal pool of chimeras was measured as a parallel by incubating cells for 2 h with 3 μg/ml iodinated TF in medium 1. The cells were washed to remove free TF, incubated at 4°C for 2 min in 0.5 mM NaCl, 0.5 M glacial acetic acid, pH 3.0, to remove TF bound to the cell surface. The cells are washed once with medium 2 (4°C), solubilized, and radioactivity measured. The percent of chimeras on the cell surface is calculated as the ratio of the Percent Surface Well to the sum of the Surface TF binding and the internal TF ( cpmWell).

Approach to Steady-state TF Occupancy—Cells were incubated for 1 h in medium 1 at 37°C in 5% CO₂ and incubated in medium 1 containing 3 μg/ml iodinated TF for various times at 37°C. At the times indicated the cells were washed three times with medium 2, solubilized, and the radioactivity determined.

Recycling Assay—Cells were preincubated for 15 to 60 min in medium 1 at 37°C in 5% CO₂, followed by an incubation in medium 1 with 3 μg/ml iodinated TF for 2 h at 37°C in 5% CO₂. The cells were washed with medium 2, incubated for 2 min in 0.5 mM NaCl, 50 mM MES, pH 5.0, followed by three washes with medium 1 supplemented with 3 μg/ml unlabelled TF. The incubation in acid results in labeled TF bound to the surface of the cell at 37°C in 5% CO₂, in medium 2 with 3 μg/ml unlabelled dipherf TF and 100 μM of the iron chelator desferroxamine. At the times indicated the medium was collected and the cells solubilized. The radioactivity in the medium is the TF released from the cells during the incubation, and the cell-associated radioactivity is the TF remaining inside cells. The recycling rate constant is the slope of the natural logarithm of the percent TF remaining cell-associated versus time.

Internalization Assay—Cells were incubated for 15 to 60 min in medium 1 at 37°C in 5% CO₂, followed by an incubation in medium 1 with 3 μg/ml iodinated TF at 37°C in 5% CO₂ for the indicated times. The cells were removed from the incubator, washed once with medium 2, placed on ice, washed twice with medium 2 (4°C), and incubated for 5 min at 4°C in 0.5 mM NaCl, 0.5 M glutathione, pH 3.0. The cells were washed 3 times with medium 2 (4°C), solubilized, and radioactivity determined by γ counting. This value represents the TF internalized during the incubation time at 37°C since incubation at 4°C in the low pH buffer will remove TF bound to the surface. To calculate the inter-
nization rate constant the amount of Tf internalized is divided by the amount of Tf bound to the surface of cells at steady-state, which is determined in a parallel by incubating cells with iodinated Tf for 2 h at 4°C. A plot of the ratio of internal to surface Tf versus time yields a straight line whose slope is the internalization rate constant.

Cytosolic Acidification—To determine the effect of cytosolic acidification on internalization, cells were incubated for 15 min at 37°C in 10 mM acetic acid, 150 mM NaCl, 1 mM CaCl2, 5 mM KCl, 1 mM MgCl2, 50 mM MES, pH 5.0. Control cells were incubated in the same buffer without acetic acid. Following the 15-min incubation the cells were placed on ice, washed three times with medium 2, and Tf binding determined by incubation with iodinated Tf for 2 h at 4°C. Inhibition of internalization through clathrin-coated pits is reflected by an increase in Tf binding to cells incubated with the buffer containing acetic acid relative to cells incubated in buffer without acetic acid (27).

Trichloroacetic Acid Precipitation—The efflux medium was made 15% trichloroacetic acid, incubated on ice for 20 min, and centrifuged (15,000 × g) for 5 min. The cell monolayers were solubilized in 1% Triton X-100 and 0.1% NaOH. The radioactivity in the solubilized cells, the trichloroacetic acid-soluble and -insoluble fractions were measured in a γ-counter. These fractions represented the internalized/retained Tf, the non-degraded/released Tf, and the degraded/released Tf, respectively.

35Fe Accumulation—Cells were incubated in medium 1 for 1 h at 37°C in 5% CO2, and then incubated in 3 μg/ml 35Fe-labeled Tf at 37°C in 5% CO2. At the indicated times cells were removed from the incubator, washed three times with medium 2, solubilized, and cell-associated 35Fe determined. The iron accumulation data are corrected for the amount of surface Tf binding, determined in parallel dishes as described above. By correcting for surface Tf binding the rates of iron-accumulation amount different cells can be compared.

Immuno-fluorescence—Cells grown on coverslips were incubated with 10 μg/ml Cy3-Tf in medium 1 at 37°C in 5% CO2 for 2 h, washed three times with medium 1, fixed for 20 min in 3.7% formaldehyde, and incubated in 1:500 dilution of B3/25 antibody (Boehringer Mannheim) in 100 μg/ml saponin in medium 2 for 1 h at 37°C. The cells were washed three times, 5 min each wash, in medium 2 with 100 μg/ml saponin and 1 mg/ml ovalbumin. The B3/25 antibody was detected by incubating the cells with a fluorescein-labeled rabbit anti-mouse IgG antibody (Cappel, Durham, NC) for 1 h at 37°C in medium 2 containing 100 μg/ml saponin. The cells were washed three times, and examined on an axiowert microscope with a 63 × objective.

RESULTS

To characterize the endocytic trafficking motifs contained in the carboxyl terminus of GLUT4, a chimera (GTCTR) was constructed in which the 30 carboxyl-terminal GLUT4 amino acids are fused to the cytosolic side of the transmembrane domain of the human TR (Fig. 1). In the GTCTR chimera the GLUT4 sequences replace the 61-amino acid cytoplasmic domain of the human TR. Previous studies have demonstrated that a di-leucine sequence (residues 489 and 490) of the carboxyl terminus plays a role in the endocytic trafficking of GLUT4 (16, 17). In the GTCTR chimera the distance of the di-leucine sequence from the membrane is 21 amino acids, similar to the 22-amino acid spacing of the di-leucine sequence from the membrane in GLUT4. It was important to conserve the spacing of the di-leucine sequence from the membrane because the spacing of endocytic trafficking motifs from the membrane is a parameter known to influence the function of endocytic trafficking motifs (28, 29). In the GTCTR chimera the polarity of the GLUT4 sequences is reversed. The polarity of the polypeptide chain with respect to the membrane is not a critical parameter for the function of many endocytic trafficking motifs (28, 29). In addition to the GTCTR chimera, mutants of GTCTR in which the di-leucine sequence was mutated to a di-alanine sequence, and mutants in which serine 488 was mutated to either an alanine or an aspartate were constructed (Fig. 1). The constructs were transfected into TRVb CHO cells which do not express functional endogenous hamster TR (25). In these cells Tf can be used to characterize the endocytic properties of the chimeras in a background free of endogenous TR. Clonal cell lines expressing the various chimeras were isolated, expanded, and the endocytic properties of the chimeras were compared to the behavior of TRVb cells expressing the wt human TR (25). TRVb-1 cells express 150,000 to 180,000 surface TR per cell (25). All the clonal lines studied expressed between 20 and 50% fewer surface Tf binding sites than TRVb-1 cells. It has previously been shown that the TR endocytic kinetic parameters are unaffected over a range of expression between 30,000 and 300,000 TR per cell (21), consequently the levels of expression of the chimeras used in this study should not affect their endocytic trafficking.

Cellular Distribution of GTCTR Chimera—Fluorescence microscopy was used to examine the steady-state distribution of the GTCTR and GTCTR-AA chimeras. To label the chimeras that are in equilibrium with the surface (that is, accessible to Tf added to the medium), cells were incubated with fluorescent Tf for 2 h, washed, and fixed. To label the total population of chimeras expressed in cells, the fixed cells were permeabilized three times with medium 2, solubilized, and cell-associated 35Fe determined and stained using a monoclonal antibody (B3/25) which recognizes an epitope on the extracellular domain of the human TR. The pattern of fluorescent Tf in cells expressing the GTCTR chimera is similar to that observed for the wt TR, with the majority of fluorescent Tf concentrated in the pericentriolar region (Fig. 2, A and C). In CHO cells recycled membrane proteins are trafficked to a pericentriolar recycling compartment by a default, bulk flow process (9). Tf accumulates here because the rate-limiting step in recycling is exit from this compartment (9, 30). The observation that Tf internalized by the GTCTR chimera is sorted to the region of the cell that contains the general recycling compartment indicates that the carboxyl-terminal sequences of GLUT4 do not divert the TR from the general recycling pathway. In cells expressing the GTCTR-AA chimera a significant fraction of the fluorescent Tf is on the plasma membrane (Fig. 2E). The Tf internalized by the GTCTR-AA chimera is delivered to the pericentriolar recycling compartment, indicating that once internalized the GTCTR-AA is trafficked to the general recycling compartment.

As is the case for the wt TR, the intracellular compartments labeled with fluorescent Tf internalized by the chimeras are also labeled with the B3/25 monoclonal antibody (Fig. 2, B, D, and F). The co-distribution of the compartments detected with the antibody and those detected with the Tf demonstrates that the chimeras are not sequestered in an intracellular compartment which is not in equilibrium with the surface. These mi-
Electron microscopy studies indicate that the steady-state distribution of GTCTR is predominantly intracellular, whereas the substitution of di-alanine for the di-leucine sequence of GTCTR shifts the steady-state distribution toward the cell surface. The finding that the distribution of the GTCTR-AA chimera is shifted toward the surface is consistent with previous reports that mutation of the di-leucine sequence shifts the distribution of GLUT4 toward the cell surface (16, 17).

Steady-state Kinetics—To measure the kinetics of GTCTR trafficking it is necessary to determine the time it takes for the chimeras inside the cells to equilibrate with chimeras on the cell surface. Cells expressing either the wt TR or GTCTR were incubated with iodinated Tf and the total cell-associated Tf was determined as a function of time (Fig. 3). The total cell-associated Tf plateaus within 2 h in cells expressing either the wt human TR or the GTCTR chimera. In all the following kinetic studies a 2-h incubation was used to label the cycling pool of the chimeras.

Next we determined the fraction of the chimeras on the cell surface at steady-state (“Materials and Methods”). At steady-state ~20% of the GTCTR chimera and ~70% of the GTCTR-AA chimera are on the surface (Fig. 4). At steady-state
35% of the wt TR on the surface (Fig. 4 and Refs. 20, 22, and 24). The increased steady-state intracellular distribution of GTCTR could be achieved by slowing the recycling of GTCTR back to the cell surface, by increasing the rate of internalization, or by changes in both parameters relative to the TR. Similarly, the shift in the GTCTR-AA toward the cell surface could result from changes in internalization, recycling, or both parameters. An advantage of using the GTCTR chimera for studies of trafficking determined by GLUT4 carboxyl-terminal sequences is that the individual kinetic rate constants can be measured.

Slowed Recycling Does Not Account for the Increased Intracellular Distribution of GTCTR at Steady State—To measure the rate at which GTCTR is recycled back to the cell surface, cells were incubated for 2 h with iodinated-Tf. Free Tf and Tf bound to the cell surface were removed, and the cells were incubated at 37 °C in medium containing unlabeled Tf and the iron-chelator desferroxamine (Materials and Methods). The data from a representative experiment are shown. The results from a clonal line expressing the wild-type human TR and clonal lines expressing either the GTCTR chimera (clones 15 and 23) or the di-alanine for di-leucine mutants of GTCTR (clones 16 and 22). These GTCTR chimeras express about one-fifth the number of chimeras as the control line expresses the wt human TR.

Fig. 3. Approach to steady-state TR occupancy with Tf in cells expressing the wt human TR or the GTCTR chimera. The results from a representative experiment are shown. Similar results were found in three other experiments. In panel A the data from a clonal line expressing the wt human TR is shown and in panel B the data from two different clonal lines expressing the GTCTR chimera are shown (clones 1 and 48). The total cell-associated radioactivity per time is presented. Each point is the average of quadruple measurements ± S.D. Note the different y axis scales in panels A and B. These GTCTR chimeras express about one-fifth the number of chimeras as the control line expresses the wt human TR.

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  <image>
  <caption>Fig. 3. Approach to steady-state TR occupancy with Tf in cells expressing the wt human TR or the GTCTR chimera. The results from a representative experiment are shown. Similar results were found in three other experiments. In panel A the data from a clonal line expressing the wt human TR is shown and in panel B the data from two different clonal lines expressing the GTCTR chimera are shown (clones 1 and 48). The total cell-associated radioactivity per time is presented. Each point is the average of quadruple measurements ± S.D. Note the different y axis scales in panels A and B. These GTCTR chimeras express about one-fifth the number of chimeras as the control line expresses the wt human TR.</caption>
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  <caption>Fig. 4. Percent of wt TR, GTCTR, and GTCTR-AA on the surface at steady-state. The results shown are the average values ± S.E. from at least three independent determinations. The fraction of Tf on the cell surface was determined as described under “Materials and Methods.” The data are from a clonal line expressing the wild-type human TR and clonal lines expressing either the GTCTR chimera (clones 1, 15, and 48) or the di-alanine for di-leucine mutants of GTCTR (clones 16 and 22).</caption>
</figure>

GTCTR Contains a Di-leucine-based Internalization Motif—The internalization rate constants of the chimeras were measured by monitoring the intracellular accumulation of Tf as a function of time (Materials and Methods). GTCTR is internalized at ~1.5 times faster than the TR, and mutation of the di-leucine sequence to a di-alanine reduces internalization of GTCTR by 10-20% more slowly than the wt TR. As is the case for cells expressing the wt TR, greater than 90% of the Tf is released after 60 min from cells expressing either the GTCTR or GTCTR-AA chimeras (not shown). The Tf released from cells had not been degraded, since greater than 90% was precipitated with 15% trichloroacetic acid (Table I). These results indicate that Tf internalized by the chimeras is returned to the cell surface and that the carboxyl-terminal domain of GLUT4 does not target the GTCTR chimera to degradative compartments (e.g. late endosomes/lysosomes). The observation that the GTCTR and GTCTR-AA chimeras are recycled at the same rate indicates that substitution of alanines for leucines 489,490 does not affect recycling of GTCTR.

To determine whether the GTCTR chimera is internalized through clathrin-coated pits, the effect of cytosol acidification on the internalization of GTCTR was examined. Cytosol acidification arrests clathrin-coated vesicle formation (31). The block in internalization results in an increase of TR on the cell surface, since recycling of internal TR to the cell surface is not affected. Acidification of the cytosol resulted in an increase in wt TR and GTCTR chimera on the cell surface, indicating that the GTCTR chimera, like the wt TR, is internalized through clathrin-coated pits (Fig. 6C).
The rate at which cells accumulate iron from diferric Tf was measured to confirm the relative rates of internalization of the wt TR and GTCTR chimera (clone 1) are shown in panel A. The data are plotted as the log of the percent Tf remaining cell-associated versus time. Each point is the average of four determinations ± S.D. In panel B the mean recycling rate constants determined in at least four independent experiments ± S.E. are presented. Two clonal lines expressing either the GTCTR chimera (clones 1 and 48) or the GTCTR-AA chimera (clones 16 and 23) are shown. The p values (unpaired two-tailed Student t test) for comparing the recycling rates of the chimeras to the wt TR are shown.

![Figure 5](image.png)

**Fig. 5.** Recycling kinetics of GTCTR and GTCTR-AA. The results from a representative recycling experiment comparing recycling of the wt TR and GTCTR chimera (clone 1) are shown in panel A. The data are plotted as the log of the percent Tf remaining cell-associated versus time. Each point is the average of four determinations ± S.D. In panel B the mean recycling rate constants determined in at least four independent experiments ± S.E. are presented. Two clonal lines expressing either the GTCTR chimera (clones 1 and 48) or the GTCTR-AA chimera (clones 16 and 23) are shown. The p values (unpaired two-tailed Student t test) for comparing the recycling rates of the chimeras to the wt TR are shown.

| Chimera expressed | % of recycled Tf that is precipitated with 15% trichloroacetic acid |
|-------------------|---------------------------------------------------------------|
| wt TR (clone TRVb-1) | 96 ± 0.1                                                      |
| GTCTR             | 93 ± 0.5                                                       |
| GTCTR-AA (clone 23) | 95 ± 2.0                                                       |

*The data presented are the averages ± S.D. of two experiments. The data for GTCTR is pooled data from two experiments on two different clonal lines expressing GTCTR. In each case greater than 93% of the Tf associated with cells at time 0 was released during the 60-min incubation.

The rate at which cells accumulate iron from diferric Tf was measured to confirm the relative rates of internalization of the GTCTR, GTCTR-AA chimeras, and wt TR (Fig. 7). Cells expressing GTCTR accumulate iron from Tf 40% more rapidly than cells expressing the wt TR, and cells expressing GTCTR-AA accumulate iron at less than 10% the rate of GTCTR. These results are consistent with the measured internalization rate constants. The observation that the rates of iron accumulation parallel the internalization rates also demonstrates that GTCTR internalizes Tf into an acidic endosomal compartment leading to dissociation of iron from Tf.

Treatment of cells expressing either the wt TR or the GTCTR chimera with 100 nM insulin for 30 min at 37 °C resulted in a modest (20%) increase in surface Tf binding (not shown). Thus, when expressed in CHO cells, the GTCTR chimera is not specifically redistributed to the plasma membrane upon insulin treatment. The relatively small increases in surface expression of GTCTR and wt TR likely reflect an effect of insulin on general membrane trafficking. A small increase in surface expression of TR following insulin treatment has been reported in a number of cell lines (8, 22).

The Measured Internalization and Recycling Rates of the Chimeras Account for the Measured Steady-state Distribution—At steady-state the distribution of membrane proteins...
between the surface and interior of cells is determined by the ratio of the recycling to internalization rate constants. To confirm the measured rates of internalization and recycling for the GTCTR and GTCTR-AA chimeras, the surface-to-internal ratio of the chimeras were measured and compared to the surface-to-internal ratio calculated as the ratio of the recycling to internalization rate constants (Fig. 8). The measured surface-to-internal ratios compare well with the ratios of recycling to internalization rate constants, demonstrating that the measured internalization and recycling rate constants determine the steady-state distribution of the GTCTR and GTCTR-AA chimeras.

Mutation of Serine 488 Slows Internalization of GTCTR—Serine 488, adjacent to the di-leucine motif of the carboxyl domain of GLUT4, is the major phosphorylation site of GLUT4 (32). It is has been proposed that hormonal regulation of serine 488 phosphorylation may be involved in regulating GLUT4's distribution between the interior and the surface of cells (33, 34). To investigate the role of the phosphorylation state of serine 488 in the trafficking of the GTCTR chimera, we mutated serine 488 to either an aspartate (S488D), to mimic the negative charge of a phosphorylated serine, or to alanine, to explore the requirement for a serine at this position (S488A).

Both the alanine and aspartate substitutions reduced the rate of GTCTR chimera internalization by 3-fold (Fig. 9A). Consistent with the reduced rate of internalization, a greater fraction of both GTCTR-S488A and GTCTR-S488D are on the surface than is the GTCTR chimera (Fig. 9B). The steady-state distributions of GTCTR-S488A and GTCTR-S488D are consistent with mutation of serine 488 slowing the rate of internalization without significantly altering the rate of recycling of GTCTR. These results demonstrate that serine 488 is an element of the di-leucine-based endocytic trafficking motif of the carboxyl terminus of GLUT4. The finding that substitution of aspartate for serine alters trafficking of GTCTR is consistent with the proposal that the phosphorylation state of serine 488 plays a role in GLUT4 trafficking. It is not possible, however, to interpret these results strictly in the context of the phosphorylation state of serine 488, since substitution of serine 488 with an alanine behaves the same as substitution with the negatively charged aspartate residue.

**DISCUSSION**

To identify the endocytic trafficking motifs of GLUT4 we have characterized the behaviors of chimeras between the TR and either the cytoplasmic amino terminus of GLUT4 (22) or the cytoplasmic carboxyl terminus of GLUT4 (this report). An advantage of this approach is that the internalization and recycling rates of the GLUT4-TR chimeras can be directly measured. The steady-state distribution of GLUT4 between the interior and cell surface is determined by the rates of internalization and recycling, therefore to understand the underlying molecular mechanisms that determine the distribution of GLUT4 it is necessary to know the extent to which internalization and recycling are regulated.

In this report we demonstrate that the carboxyl cytoplasmic domain of GLUT4 contains a serine-leucine-leucine-based internalization motif. The best characterized membrane protein trafficking motifs are based on aromatic amino acids (28, 29). There are, however, a number of examples of non-aromatic amino acid-based trafficking motifs, specifically motifs containing leucine residues (35, 36). Both classes of motifs have been shown to mediate rapid internalization from the plasma membrane. Because the rate of internalization reflects the degree of clustering, it is likely that the GTCTR chimera is more efficiently clustered in coated pits than is the wt TR (29). At this time it is not known whether the more rapid rate of internal-
GLUT4 C-terminal SLL Internalization Motif

Fig. 9. Internalization kinetics of GTCTR, GTCTR-S488A, and GTCTR-S488D chimeras. In panel A the mean internalization rate constants for the GTCTR-S488A (clone 48) or GTCTR-S488D (clone 3) are shown. The values are the means ± the S.E., n > 4. The result for GTCTR (clone 48) are from Fig. 6B and are shown to illustrate the effect of the serine 488 mutations on internalization of GTCTR. In panel B the fraction of the GTCTR-S488A (clone 48) and GTCTR-S488D (clone 3) on the cell surface at steady state are shown. The values are the means ± S.E., n ≥ 4. The results for the GTCTR chimera (clone 48) are from Fig. 3 and are shown here to illustrate the shift in the distribution of GTCTR caused by the serine 488 mutations.

Internalization promoted by the GLUT4 serine-leucine-leucine-based internalization motif is a characteristic of the di-leucine-based motifs or is specific to GLUT4 sequences.

Many of the leucine-containing motifs, in addition to promoting internalization, target proteins to late endosomes and lysosomes by diverting them from the general recycling pathway (35–38). Two experiments suggest that the serine-leucine-leucine-based motif of GLUT4 does not divert the GTCTR chimera from the bulk recycling pathway in CHO cells. First, GTCTR traffics through the same morphologically defined endosomal recycling compartments as does the wt TR. Second, Tf internalized into cells by GTCTR is recycled back to the medium intact, demonstrating that the released radioactivity is not due to degradation of Tf, as would be the case if Tf was targeted to lysosomes.

When expressed in CHO cells GLUT4 is distributed such that ~80% is inside cells at steady-state (12, 18). We find ~80% of the GTCTR chimera is inside cells at steady-state, thereby demonstrating that the carboxyl cytoplasmic domain of GLUT4, in the absence of other endocytic trafficking motifs of GLUT4, is sufficient to promote the degree of intracellular concentration observed for authentic GLUT4. Mutation of the di-leucine sequence of GLUT4 or GTCTR shifts their distributions toward the surface when expressed in non-insulin responsive cells, indicating that the carboxyl cytoplasmic domain of GLUT4 functions similarly in the GTCTR chimera as it does in intact GLUT4 (16, 17). It has been reported that mutation of the di-leucine sequence affects GLUT4’s distribution by reducing internalization (17). Our results are in agreement with the conclusions of that study. Furthermore, in a direct comparison with the kinetics of TR trafficking, we have shown that it is the quantitative difference in the rates of internalization promoted by the serine-leucine-leucine-based motif of GLUT4 and the YTRF motif of TR that determines the greater steady-state intracellular concentration of GTCTR.

In insulin-responsive cells GLUT4 is recycled at one-fifth to one-tenth the rate of other membrane proteins, and it is this slowed recycling that results in the pronounced intracellular concentration of GLUT4 (6, 7). Neither the carboxyl terminus of GLUT4 (this report) nor the amino terminus of GLUT4 (22) contain information that regulate recycling in non-insulin responsive cells (22). The requirement for cell-type specific factors for insulin-regulated GLUT4 trafficking may explain the failure to identify the GLUT4 domains that regulate recycling.

Regardless, studies of GLUT4 trafficking in non-insulin responsive cells have yielded important information. GLUT4 is internalized through clathrin-coated pits in insulin-responsive cells (39), therefore the serine-leucine-leucine-based motif of the carboxyl terminus and/or the phenylalanine-based motif of the amino terminus of GLUT4, identified in studies of non-insulin responsive cells, are likely to regulate GLUT4’s internalization in insulin-responsive cells. These two motifs promote different rates of internalization. The phenylalanine-based motif promotes internalization at one-third the rate of the serine-leucine-leucine-based motif. The different rates of internalization determined by these motifs may play a role in the insulin-induced increase of GLUT4 on the cell surface. One effect of insulin on GLUT4 trafficking is to slow internalization by 30–70% (6, 7, 10). Modulation of GLUT4’s internalization may reflect which motif determines the rate of internalization in the basal and insulin-stimulated states. In addition, motifs that determine specific intracellular targeting of membrane proteins are closely related to internalization motifs (28, 29). Thus, it is possible that in addition to functioning as internalization motifs, the serine-leucine-leucine-based and/or phenylalanine-based motifs may also function to regulate recycling of GLUT4 by interacting with cell-type specific factors expressed in insulin-responsive cells.

Three recent studies have examined the distributions of GLUT4 constructs in insulin-responsive cell types (40–42). In two studies a role for GLUT4’s amino terminus in trafficking in insulin-responsive cells was established (40, 41) and in one study the requirement for the phenylalanine of the amino-terminal phenylalanine motif was demonstrated (40). Mutation of this phenylalanine to alanine shifted the basal state distribution of the GLUT4 toward the surface. However, it is not known whether this shift in distribution is a result of decreased internalization, increased recycling, or changes in both parameters. All three studies agree that the carboxyl-terminal domain contains important trafficking information. Mutation of the di-leucine sequence of the carboxyl domain delays the re-establishment of the basal-state distribution following removal of insulin (41). An interpretation of this finding is that the di-leucine sequence functions as to promote rapid internalization, a prerequisite for the re-targeting of GLUT4 from the plasma membrane to the site of intracellular storage. This is consistent with our findings that the serine-leucine-leucine sequence is an effective internalization motif. The results of all these studies agree that information in the carboxyl domain outside of the di-leucine sequence is required for insulin-regu-
lated GLUT4 trafficking. These studies in insulin-responsive cells indicate the need for kinetic studies of the trafficking of GLUT4 constructs in insulin-responsive cells. A future objective of our work is to kinetically characterize the endocytic behavior of the GLUT4-TR chimeras in insulin-responsive cells, in order to identify the functions of these domains in determining GLUT4’s distribution in the basal and insulin-stimulated states.

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