The Amino Terminus of GLUT4 Functions as an Internalization Motif but Not an Intracellular Retention Signal When Substituted for the Transferrin Receptor Cytoplasmic Domain

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Abstract. Previous studies have demonstrated that the amino-terminal cytoplasmic domain of GLUT4 contains a phenylalanine-based targeting motif that determines its steady state distribution between the surface and the interior of cells (Piper, R. C., C. Tai, P. Kuleza, S. Pang, D. Warnock, J. Baenziger, J. W. Slot, H. J. Geuze, C. Puri, and D. E. James. 1993. J. Cell Biol. 121:1221). To directly measure the effect that the GLUT4 amino terminus has on internalization and subsequent recycling back to the cell surface, we constructed chimeras in which this sequence was substituted for the amino-terminal cytoplasmic domain of the human transferrin receptor. The chimeras were stably transfected into Chinese hamster ovary cells and their endocytic behavior characterized. The GLUT4-transferrin receptor chimera was recycled back to the cell surface with a rate similar to the transferrin receptor, indicating that the GLUT4 sequence was not promoting intracellular retention of the chimera. The GLUT4-transferrin receptor chimera was internalized at half the rate of the transferrin receptor. Substitution of an alanine for phenylalanine at position 5 slowed internalization of the chimera by twofold, to a level characteristic of bulk membrane internalization. However, substitution of a tyrosine increased the rate of internalization to the level of the transferrin receptor. Neither of these substitutions significantly altered the rate at which the chimeras were recycled back to the cell surface. These results demonstrate that the major function of the GLUT4 amino-terminal domain is to promote the effective internalization of the protein from the cell surface, via a functional phenylalanine-based internalization motif, rather than retention of the transporter within intracellular structures.

FACILITATED transport of glucose is mediated by a family of 12 membrane-spanning glycoproteins. There are six functional facilitative glucose transporter protein isoforms (GLUTs) known in man (reviewed in James et al., 1993; Bell et al., 1993). The human isoforms are ~500 amino acids in length and share ~50–75% sequence identity. In insulin-responsive cells (i.e., adipose, skeletal, and heart muscle) insulin stimulates the rapid translocation of the major insulin-responsive transporter (GLUT4) from intracellular storage sites to the plasma membrane (Cushman and Wardzala, 1980; Suzuki and Kono, 1980). In these cells the 10–40-fold increase in the surface expression of GLUT4 is accompanied by a proportionate surge in glucose transport (Holman et al., 1990; Slot et al., 1991). Cessation of the insulin signal marks the resumption of the primarily intracellular localization of GLUT4. In 3T3-L1 adipocytes, 90–99% of GLUT4 is excluded from the cell surface in the absence of insulin (Piper et al., 1991; Jhun et al., 1992; Yang and Holman, 1993). Furthermore, the intracellular sequestration of GLUT4 is preserved when this isoform is expressed in heterologous cells such as 3T3-L1 fibroblasts or HepG2 cells (Haney et al., 1991), CHO cells (Asano et al., 1992; Piper et al., 1992), COS-7 cells (Czech et al., 1993), and PC12 cells (Verhey et al., 1993). The property of sequestration is not a characteristic of the other glucose transporters. GLUTs 1, 2, and 3 are all randomly targeted to the plasma membrane in a variety of cell types (reviewed in James et al., 1993). GLUT1 is presumed to function in the basal (or constitutive) transport of glucose based upon its predominant plasma membrane location and broad tissue distribution. Therefore, GLUT4 is thought to contain unique targeting domains that encode for its intracellular sequestration.

In the absence of insulin, GLUT4 continuously cycles between the plasma membrane and intracellular compartments suggesting that the apparent intracellular sequestration of GLUT4 reflects the steady state distribution of the trans-
mutagenesis highlighted the NH2-terminal amino acids by deleting the NH2-terminal eight amino acids of GLUT4 cellular to a predominantly surface distribution was achieved (et al., 1992). A similar change from a predominantly intra-
tele sequence homology between GLUT isoforms. It was et al., 1993; Verhey et al., 1993). The amino- and carboxy-
tify the region(s) of GLUT4 that are responsible for its char-
GLUT4 is currently the subject of intense investigation.
In particular, the aromatic amino acid was a critical
minus of GLUT4 with that of GLUT1 yielded a chimera
GLUT1 with that of GLUT4 caused a large increase in intra-
cellular sequestration. Conversely, replacing the amino ter-
minus of GLUT4 with that of GLUT1 yielded a chimera
which was preferentially located on the cell surface (Piper et al., 1992). A similar change from a predominantly intra-
cellular to a predominantly surface distribution was achieved
by deleting the NH2-terminal eight amino acids of GLUT4 (Piper et al., 1993). Further studies using alanine-scanning mutagenesis highlighted the NH2-terminal amino acids PSGFQIQ (2-8) as containing important targeting informa-
tion. In particular, the aromatic amino acid was a critical component of this motif because mutation of the phenylala-
nine alone to alanine caused a large increase in cell surface expression. An important function for this motif in regulat-
ing the entry of GLUT4 into cell surface clathrin-coated pits was evident because, whereas GLUT4 colocalized
with clathrin lattices in CHO cell plasma membranes, no such colocalization was evident for the GLUT4 alanine for
phenylalanine-5 mutant. It was further shown that the amino
terminus of GLUT4, when transferred to another protein,
could confer a preferentially intracellular distribution to a heterologous protein (Piper et al., 1993). One interpretation
of these findings was that the GLUT4 amino terminus con-
tains an intracellular retention motif. However, the alter-
native explanation that the GLUT4 sequence was increasing
internalization without slowing recycling and thereby achieving a predominantly intracellular distribution was not ruled out (Piper et al., 1993). The heterologous protein used in the above studies was the H1 subunit of the asialoglycoprotein receptor which does not bind ligand in the absence of the H2 subunit. Due to technical limitations of these systems, it was not possible to accurately measure recycling kinetics for either GLUT4-GLUT1 chimeras or GLUT4-heterologous protein chimeras in previous studies. Thus, it remained to be determined which aspect of GLUT4 trafficking, internalization, or recycling, was being affected by the NH2-terminal sequence.

To allow us to directly address which aspects of protein trafficking are being affected by the GLUT4-targeting motif, we have replaced the 61-amino acid cytoplasmic domain of the human transferrin receptor (TR) with the 19–amino acid, NH2-terminal domain of GLUT4. In these constructs, the portion contributed by the TR acts as a reporter molecule for the cellular distribution determined by the GLUT4 sequence. The endocytic behavior of the chimera was characterized in stably transfected CHO cells. We have quantified the steady state distribution of the chimera between intracel-
ular pools and the plasma membrane. More importantly, the individual rates of chimera internalization and externaliza-
tion (rates which determine the steady state distribution of membrane proteins) were also measured. Our analysis has revealed that the 19–amino acid, NH2-terminal cytoplasmic sequence of GLUT4 does not cause selective retention of the chimera within CHO cells, but rather promotes efficient in-
ternalization of the chimera in a manner critically dependent upon the residue at position 5.

Materials and Methods

Cell Culture

All cells used in this report were carried in either Ham's F12 medium or McCoy's SA medium containing 5% FBS, 2% penicillin-streptomycin (GIBCO BRL, Gaithersburg, MD), and 220 mM sodium bicarbonate (Sigma Immunochemicals, St. Louis, MO). Cells were grown at 37°C in a humidified atmosphere of 5% carbon dioxide in air. The chimeric constructs were transfected into TRVb cells, a variant CHO cell line that does not express functional, endogenous hamster TR (McGraw et al., 1987). These cells have been used for a number of previous studies of the endocytic behavior of the TR and mutants of the TR (e.g., McGraw et al., 1991). The endocytic properties for the chimeras were compared to TRVb cells expressing either the wild-type human transferrin receptor (TRVb-l; McGraw et al., 1988) or to TRVb cells expressing a mutant receptor (Δ3-59 TR) in which the cytoplasmic domain has been deleted (Johnson et al., 1993). The endocytic characteristics of TRVb-l cells have been extensively character-
ized (e.g., Dunn et al., 1989; McGraw and Maxfield, 1990; Mayor et al., 1993).

Ligands

Human transferrin (Tf) was obtained from Sigma Immunochemicals and further purified by Sepharyl S-300 gel filtration. Diferric Tf and 55Fe2-Tf were prepared as described previously (Yamashiro et al., 1984). 55FeCl3 was purchased from New England Nuclear (Cambridge, MA) and 55Fe2-Tf was prepared by the nitritiociac acid method (Klausner et al., 1984). In-
sulin (Sigma Immunochemicals) was reconstituted fresh the day of the ex-
periment. Tf was labeled with Cy3 reactive dye (Biological Detection Sys-
tems, Pittsburgh, PA) according to the manufacturer's instructions.

cDNA Constructions

DNA manipulations were performed according to standard techniques.
the NHe-terminal 19 amino acids of GLUT4 to the transmembrane region cloned into pUC8) (McGraw et al., 1988) were used. The scheme linking cDNA (coding a tyrosine for phenylalanine in the 5 position of GLUT4) constructed by PCR. To construct chimera GTTR-Y, F-Y plRGT shuttle previously published. Restriction enzymes and T4 DNA ligase were pur-

sbrook et al., 1989). The CDNA sequences of GLUT4 (Birnbaum, and the following oligonudcotide which has a 16-bp overlap with the sequence of the TR sequence: 5'GCAGATACTTCCACTACATCGC'1UCTGAGG-

the full chimeric GTTR cDNAs was confirmed by dideoxy sequencing in Fig. 1. The GTTR-F and GTTR-A chimeras were constructed by oligonucleotide to the TR sequence: 5"GCAGATACTTCCACTACATCGC'1UCTGAGG-

and 3 ttg/ml 125I-Tf)for 2 h. Five washes with chilled Medl preceded incubation media removed. Cell monolayers were flooded with ice-cold harsh acid wash stripping of the surface bound Tf. In each case, a vehicle-treated cluster of cells was run in parallel.

**Insulin Stimulation**

When insulin was used, cell monolayers were preincubated in McBB (2 mg/ml ovalbumin) for 2 h to remove any growth factors present in the growth medium supplemented with 5% FBS. In experiments involving insulin up-regulation of surface Tf, 100 nM of the hormone was added to wells prior to the addition of 125I-Tf as above. For experiments determining insulin's effect on the internal pool of Tf, 100 nM of the hormone was added to the wells 105 min after the addition of 125I-Tf as above, yet 15 min before ice-cold harsh acid wash stripping of the surface bound Tf. Following 2 Medl washes, the cells were solubilized and the gamma radioactivity was counted. In parallel dishes, the number of surface TRs was measured as above. At steady state, the ratio of surface TR to internal TR reflects the ratio of the rate constants for externalization to internalization.

**Iron Accumulation**

The ability of cells to accumulate iron from differic-Tf was assayed as previously described for 59Fe (McGraw and Maxfield, 1990). Specific 59Fe accumulation was determined by subtracting the nonspecific 59Fe-Tf bound (in the presence of a 50-fold excess Tf) from the cell-associated 59Fe. The surface pool of TR was measured in parallel dishes by incubating with 3 

**Fluorescence Microscopy**

For Tf uptake studies and the screening of clones that had survived G418 selection, cells were washed and incubated with McBB (10 mg/ml fluorescently labeled TF, 2 mg/ml ovalbumin) for 90 min at 37°C in 5% CO2. Cells were washed with Medl to remove unbound Tf and fixed in 3.7% formaldehyde for 20 min. For indirect immunofluorescence studies, cells were permeabilized with 100 mg/ml saponin (Sigma Immunochemicals) during each 45-min incubation period with the primary and secondary anti-
bodies. The monoclonal anti-human TR antibody B3/25 was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Fluorescein-conjugated rabbit anti-mouse polyclonal antibody was obtained from Cappel (West Chester, PA). For microscopy, 35-mm cover slip dishes were imaged using a Zeiss Axiovert 35 microscope equipped with standard rhodamine/fluorescein filters and photographed using Kodak TMY 400 film.

Results

The constructs analyzed in this study are depicted in Fig. 1. In the GLUT4-TR construct (GTTR-F) the 61-amin acid cytoplasmic domain of the TR has been replaced with the 19-amin acid NH2 terminus of GLUT4. Previous studies have indicated that the phenylalanine at position 5 is critical for establishing the distribution of GLUT4 within cells (Piper et al., 1992, 1993). Thus, constructs in which a tyrosine (GTTR-Y) or alanine (GTTR-A) were substituted for wild-type phenylalanine 5 were also made (Fig. 1). Chimeric cDNAs were transfected into a variant CHO line (TRVb) that is devoid of functional endogenous hamster TR (McGraw et al., 1987). Expression of the GTTR chimeras in TRVb cells allowed for the biochemical and morphological characterization of the endocytic behavior of the chimeras in a background free of endogenous Tf binding (McGraw et al., 1987). The endocytic properties of GTTRs were compared to those measured for TRVb cells transfected with either the wild-type human TR, i.e., TRVb-1 cells (McGraw et al., 1987) or to TRVb cells expressing a mutant receptor (Δ3-59 TR) in which the cytoplasmic domain has been deleted (Johnson et al., 1993). Tf endocytosis in TRVb-1 cells has been extensively characterized using both biochemical and morphologic techniques (Dunn et al., 1989; McGraw and Maxfield, 1990; Mayor et al., 1993).

The clonal cell lines used in this study expressed a similar range of surface Tf-binding sites (40,000-120,000/cell), that was comparable to that expressed in TRVb-1 cells. It has been previously shown that over a range of TR expression between 30,000 and 200,000/cell in CHO cells, the kinetic parameters as well as the subcellular distribution of receptors remained constant (McGraw and Maxfield, 1990). This finding indicates that none of the trafficking steps are saturated within this range of TR expression, and, therefore, the level of chimera expression in this study is not a confounding factor. We have analyzed several different clones for each chimera to avoid potential contributions from clonal variation. Scatchard analysis of surface Tf binding revealed that the affinity of the chimeric receptors for Tf was not significantly different from the wild-type TR affinity for Tf (data not shown). This is consistent with previous findings that mutations of the TR cytoplasmic domain do not alter the affinity of the extracellular high-affinity Tf-binding domain of the receptor (McGraw et al., 1988; Jing et al., 1990; McGraw and Maxfield, 1990).

Fraction of the GTTR Chimeras on the Cell Surface

The fraction of the GTTR chimeras on the cell surface at steady state was determined as described in Materials and Methods. To aid in our analysis of the role that the GLUT4 NH2-terminal sequence plays in targeting, a TR construct in which the cytoplasmic domain has been deleted (Δ3-59 TR) was used as a control. The endocytic behavior of this construct has previously been described (Jing et al., 1990; Johnson et al., 1993). The Δ3-59 TR is internalized at approximately one-fifth the rate of the wild-type TR, however, once internalized the Δ3-59 TR is recycled back to the plasma membrane at the same rate as the wild-type TR. Hence, the Δ3-59 TR displays a predominantly surface distribution. The GTTR chimeras in the present study are essentially the Δ3-59 TR with the GLUT4 sequences attached at the amino terminus. Therefore, comparison of the Δ3-59 TR and the GTTR constructs allowed us to determine the role that the GLUT4 sequences play in trafficking.

The results of the steady state surface analysis are shown in Fig. 2. Approximately 50% of the GTTR-F chimera was on the surface as compared to ~70% for the Δ3-59 TR. Hence, the wild-type GLUT4 amino-terminal cytoplasmic domain contributed the distribution of the Δ3-59 TR in a fashion consistent with the hypothesis that this GLUT4 sequence contains an intracellular sequestration motif. Substitution of alanine (GTTR-A) for phenylalanine-5 increased the cell surface expression to ~70% (that is, the level observed for the Δ3-59 TR), whereas substitution of tyrosine (GTTR-Y) for phenylalanine-5 decreased the cell surface expression to ~30% (that is, the same level observed for the wild-type TR, TRVb-1). Therefore, the amino acid at position 5 of the GLUT4 sequence is critical for determining the cellular distribution of the GTTR chimeras. These results are consistent with the hypothesis that the NH2 terminus of GLUT4 contains an intracellular sequestration signal that distinguishes it from the predominant surface expression of the constitutive glucose transporter, GLUT1 (Piper et al., 1993).

GTTR Chimeras Are Recycled at a Similar Rate to the Wild-type TR

The fraction of the GTTR chimeras on the surface is dependent upon the rate at which the chimeras are internalized and recycled back to the plasma membrane. To directly investigate the ability of the amino terminus of GLUT4 to slow recycling (i.e., function as a retention motif) we measured the rates at which the chimeras were returned to the cell surface. If the GLUT4 sequence was serving as a retention signal, then the GTTR-F chimera would be more slowly recycled than native TR and the slowed recycling would be dependent upon the amino acid at position 5. Furthermore, based upon the surface distribution of GTTR-A in Fig. 2, it was predicted that the substitution of an alanine for phenylalanine-5 would inactivate a retention signal.
Garippa et al. GLUT4 NH₂ Terminus Contains an Internalization Motif

Figure 2. Percentage of total cellular GTTR chimeras expressed on the cell surface at steady-state. Values were calculated as described in Materials and Methods. TRVb-1 is a clonal line of TRVb cells expressing the wild-type human TR. GTTR-F, GTTR-A, and GTTR-Y are chimeras between GLUT-4 and the TR. The Δ3-59 TR is a construct in which all but 4 amino acids of the TR cytoplasmic domain are deleted, and has been included here for the sake of comparison. Two clonal lines of TRVb cells expressing each chimera were examined, and are labeled as -1 and -2. Shown are the mean ± SEM, based on at least three determinations.

Figure 3. Recycling of the GTTR-F chimera and human TR expressed in TRVb CHO cells. Shown are the results from a representative experiment to measure the rate at which Tf is released from cells as described in Materials and Methods. Each time point is the average of four determinations ± SD, corrected for nonspecific uptake. A summary of the externalization rate constant measurements for each cell line is presented in Table I. The amount of total cell-associated Tf in this experiment was ~9,000 and ~29,000 cpm for GTTR-F and TRVb-1, respectively.

The above externalization rate constant is a measure of the initial rate of receptor externalization (0–15 min). The efflux of Tf was examined over longer time intervals to establish that all of the 125I-Tf internalized during the 2-h preincubation period was in equilibrium with the rapid recycling pool and hence, that a fraction of the 125I-Tf was not trafficked by the GTTR chimeras to an intracellular vesicle pool separate from the one in rapid equilibrium with the cell surface. In all the chimeric cell lines, >95% of the cell-associated Tf was released into the medium by 1.5 h (data not shown). This result demonstrates that the 125I-Tf internalized by the different GTTR chimeras is delivered to compartments from which it is rapidly recycled, as is the case for 125I-Tf internalized by the wild-type TR or the Δ3-59 TR. These findings argue that there is not a significant fraction of the GTTR chimeras internalized into an intracellular compartment separate from the one rapidly returned to the cell surface. This result, together with the above measurement of the recycling rate constant, demonstrates that the GLUT4 amino-terminal cytoplasmic domain alone was not sufficient to mediate intracellular retention in that the GTTR chimeras were not recycled more slowly than the wild-type TR or the truncated TR in CHO cells.

Table I. Comparison of Exocytic Rate Constants

| Cell type | ke (min⁻¹)± SE | Percent of wild type |
|-----------|----------------|----------------------|
| TRVb-1    | 0.063 ± 0.002  | 100                  |
| GTTR-F-1  | 0.063 ± 0.002  | 100                  |
| GTTR F-2  | 0.056 ± 0.004  | 89                   |
| GTTR-A-1  | 0.050 ± 0.002  | 79                   |
| GTTR-A-2  | 0.056 ± 0.003  | 89                   |
| GTTR-Y-1  | 0.044 ± 0.003  | 70                   |
| GTTR-Y-2  | 0.048 ± 0.003  | 76                   |

* Values are the mean ± SE for a minimum of three determinations from the assays represented in Fig. 3.
The finding that differing fractions of the GTTR chimeras were found on the cell surface despite having similar recycling rates suggested that the difference in the steady state cellular distribution of the chimeras was determined by the rate at which the chimeras were internalized. The presence of an aromatic amino acid on the cytoplasmic domain of a number of receptors has been shown to be critical for establishing the rate of internalization (Davis et al., 1987; Lobel et al., 1989; Breitfeld et al., 1990; Kistakis et al., 1990; McGraw and Maxfield, 1990). As has been previously noted, the FQFI domain (amino acids 5 to 8) in the GLUT4 amino terminus fulfills the essential structural features of several known aromatic amino acid–containing internalization signals (Piper et al., 1993). To examine the rate at which the GTTR chimeras were internalized, the rate of Tf internalization was measured.

The internalization rate represents the fraction of surface GTTR internalized per minute. This constant was measured as described in Materials and Methods. As shown in Fig. 4 and Table II, the chimeras could be grouped into three distinct classes based on internalization rate constants. The GTTR chimera with a phenylalanine at position 5 had an internalization rate constant of 0.060 min⁻¹; with an alanine at position 5 the internalization rate constant was reduced to 0.025 min⁻¹; and with a tyrosine at position 5 the internalization rate constant was 0.120 min⁻¹. These values stand in comparison to the internalization rate of 0.110 min⁻¹ measured for the wild-type TR. The internalization rate constant for the GTTR-Y chimera is comparable to the rate at which the wild-type TR is internalized, a rate reminiscent of efficient internalization through clathrin-coated pits. The internalization rate constant for the GTTR-A chimera is comparable to the rate at which the Δ3-59 TR is internalized, 0.020 min⁻¹ (Johnson et al., 1993) a rate that most likely reflects bulk membrane endocytosis. Therefore, the amino-terminal cytoplasmic domain of GLUT4 controls the cellular distribution of the GTTR chimeras by modulating the rate of internalization rather than the recycling rate. Furthermore, these results suggest that the phenylalanine in the amino terminus of GLUT4 presents a less efficient internalization motif than when a tyrosine is substituted, yet a more efficient internalization signal than when an alanine is substituted.

Table II. Comparison of Endocytic Rate Constants

| Cell type | ki (min⁻¹)* SE | Percent of wild type |
|-----------|---------------|---------------------|
| TRVb-1    | 0.110 ± 0.006 | 100                 |
| GTTR-F-1  | 0.059 ± 0.006 | 53                  |
| GTTR-F-2  | 0.063 ± 0.005 | 57                  |
| GTTR-A-1  | 0.024 ± 0.001 | 22                  |
| GTTR-A-2  | 0.027 ± 0.007 | 25                  |
| GTTR-Y-1  | 0.123 ± 0.013 | 112                 |
| GTTR-Y-2  | 0.124 ± 0.012 | 112                 |

* Values are the means ± SE for a minimum of three determinations from the assay represented in Fig. 4.

The Amino Acid at Position 5 of the GTTR Chimeras Determines the Rate at Which the Chimeras are Internalized

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Measured Steady State Distribution of GTTR Chimeras Agrees with Measured Internalization and Recycling Rate Constants

At steady state, the distribution of membrane proteins between intracellular compartments and the cell surface is determined by the ratio of the exocytic rate constant to the endocytic rate constant: [S] × ki = [I] × ke or [S]/[I] = ki/ke; where [S] is surface, [I] is intracellular, and ki and ke are the internalization and externalization rate constants, respectively. Thus, the ratio of surface to internal GTTR provides an independent assessment which should reflect the ratio of the measured rate constants.

The surface/internal (S/I) ratio of the GTTR chimeras was measured as described in Materials and Methods. The standard deviation of the ke/ki ratio was determined using an alternative formula for the sample estimate of variance as previously described (Cochrane, 1977). The measured S/I ratios for the chimeras were in good agreement with the values predicted by the ratios of the rate constants, ki and ke, all falling within the standard deviation for the combined ratios (Table III). This analysis demonstrates that the measured initial rate constant reflects the steady state distribution of the various constructs and confirms the findings that all the chimeras are recycled at similar rates but internalized at markedly different rates.

Insulin Treatment Increases Surface Expression of the GTTR Chimeras and the Wild-type TR to the Same Degree

A unique characteristic of the GLUT4 transporter in fat and muscle cells is its ability to undergo rapid translocation to the plasma membrane upon insulin stimulation (Cushman and Wardzala, 1980; Suzuki and Kono, 1980). To determine whether the insulin-translocatable feature of GLUT4 could be reproduced in the GTTR-F chimera, cells were incubated with 100 nM insulin for 15 min and the number of surface Tf-binding sites was measured by incubation with 125I-Tf as described in Materials and Methods. This insulin treatment resulted in modest increases in the number of surface-binding sites (Δ12%) for the GTTR-F chimera and (Δ40%) for the TRVb-l cells. A small increase in the surface expression of TR following insulin treatment in a variety of cell lines has previously been reported and reflects an insulin-

Table III. Distribution of TR and GTTR between the Cell Surface and Interior: Comparison of Measured vs. Predicted S/I Values

| Cell type | S/I* | SE | ke/ki| SE |
|-----------|------|----|-----|----|
| TRVb-1    | 0.50 ± 0.02 | 0.57 ± 0.13 |
| GTTR-F-1  | 0.99 ± 0.08 | 1.07 ± 0.23 |
| GTTR-F-2  | 0.96 ± 0.02 | 0.88 ± 0.22 |
| GTTR-A-1  | 1.87 ± 0.09 | 2.07 ± 0.30 |
| GTTR-A-2  | 2.20 ± 0.05 | 2.08 ± 0.88 |
| GTTR-Y-1  | 0.52 ± 0.04 | 0.36 ± 0.13 |
| GTTR-Y-2  | 0.52 ± 0.03 | 0.39 ± 0.10 |

* The measured S/I ratio is expressed as the mean ± SE of a minimum of three separate experiments.

† The predicted steady state S/I ratio as calculated from the measured values for the efflux (ke) and the internalization (ki) rate constants. Each rate constant represents the mean ± SE for a minimum of three determinations.
stimulated increase in a recycling limb of the endocytic pathway that is common to a number of other receptors (Tanner and Lienhard, 1987; Corvera et al., 1989). As stated previously, the change in surface expression of GLUT4 following insulin-stimulated release is at least 10-fold. The greater increase in surface expression for TRVb-1 relative to GTTR-F reflects the fact that, prior to insulin treatment, a greater fraction of the total cellular TR for TRVb-1 is in an intracellular pool than is the GTTR-F (~70 and 50%, respectively). Thus, an equivalent increase in the rate of recycling for GTTR-F and TRVb-1 would result in a larger increase in the percentage of TR on the cell surface for TRVb-1. Our results do not demonstrate a preferential insulin-mediated mobilization (from internal to surface) of the GTTR-F chimera over the wild-type TR, suggesting the necessity for other cellular mechanisms or additional regions of the GLUT4 molecule in this process.

**GTTR Chimeras Are Capable of Iron Accumulation**

The biological function of the TR is to deliver nutritionally required iron. Thus, a method for examining the functional behavior of the GTTR chimeras was to assay the cellular $^{55}$Fe accumulation from diferric Tf. It was predicted that the differences in Tf internalization seen among the GTTR chimeras would be reflected in the rate of $^{55}$Fe accumulation.

As was observed with the internalization rates (Fig. 4), $^{55}$Fe accumulation correlated with the amino acid at position 5 of the GLUT4 NH$_2$ terminus and provided a clear distinction between the chimeras (Fig. 5). The GTTR chimera with an alanine at position 5 demonstrated the least efficient rate of iron uptake while the GTTR chimera with a tyrosine at position 5 exhibited the most efficient rate of iron uptake, comparable to the wild-type TR. The GTTR chimera with the endogenous phenylalanine in the 5 position demonstrated a rate of iron uptake that was intermediate to the other two chimeras. This result demonstrates that the GTTR chimeras are fulfilling the biological role of the TR and demonstrate that the Tf internalized by these chimeras is trafficked through intracellular compartments from which iron can be released and accumulated by CHO cells.

**Pericentriolar Location of the Endocytic Recycling Compartment Is Unaltered in GLUT4-TR Chimeras**

To ascertain whether the GTTR chimeras are trafficked through the same intracellular compartments as TR, the morphology of the Tf-labeled compartments in cells expressing the GTTR chimeras was compared to that of cells expressing the wild-type TR (TRVb-1 cells). In TRVb-1 cells, the principal internal compartment visualized by steady state labeling with fluorescent-Tf was concentrated near the microtubule-organizing center (Fig. 6a). This pericentriolar recycling compartment has been previously described as a prominent single patch of fluorescence (Yamashiro et al., 1984; Dunn et al., 1989). Endocytosed Tf is rapidly delivered to the recycling compartment (Dunn et al., 1989) and movement from this compartment back to the cell surface is the rate-limiting step in the recycling of Tf (Mayor et al., 1993). Endocytosed molecules targeted for lysosomal degradation do not traverse this compartment, but rather, they are sorted via early sorting endosomes to the degradative pathway (Dunn et al., 1989). When this was visualized by EM, the recycling compartment appeared as small vesicles and tubules $\sim$60 nm in diameter (Yamashiro et al., 1984).

As shown in Fig. 6, the recycling compartment was apparent in cells expressing any of the three GTTR chimeras. In cells expressing GTTR-F, the Tf uptake pattern and the anti-TR antibody labeling were similar to cells expressing the wild-type TR, although the increased surface expression of GTTR-F was not readily apparent in the photomicrograph. Morphologically, there was no evidence of an abnormal intracellular compartment in the GTTR-F cells. In the GTTR-A clones, the signal intensity within the recycling compartment was not as bright as in TRVb-1, GTTR-F, or GTTR-Y clones, although the fluorescent-Tf signal on the surface of the GTTR-A cells was highly prominent. This morphology is consistent with the high surface/internal ratio (Table III) measured for the GTTR-A chimeras. There were no in-
Figure 6. Fluorescent localization of stably transfected cells expressing human TR (A and B), GTTR-F (C and D), GTTR-A (E and F), or GTTR-Y (G and H). A, C, E, and G are the steady state patterns of internalized fluorescent Tf. B, D, F, and H are the same fields of cells shown in A, C, E, and G, stained with an antibody to the ectodomain of the human TR (B3/25; Boehringer-Mannhein). The recycling compartment is indicated by the arrows in E and F.
stances of an irregularly shaped recycling compartment or a preponderance of punctate structures in the GTTR chimeras. These morphologic studies imply that Tf internalized by the GTTR chimeras is trafficked through the same compartments as Tf internalized by authentic TR. Importantly, there is complete colocalization of internal compartments labeled by either endocytosis of fluorescent Tf or by B3/25 monoclonal antibody staining of the chimeras in detergent permeabilized cells. This result argues for the existence of one homogeneous recycling pool of GTTR chimeras and eliminated the possibility that there was a significant internal pool of GTTR chimeras not accessible to internalized Tf. Thus, our morphological observation using fluorescent Tf probes validates the use of steady state measurements in assessing the distribution and trafficking of the GTTR chimeras.

Discussion

We used chimeras between the GLUT4 and TR to examine the targeting information contained within the amino-terminal cytoplasmic domain of GLUT4. In these constructs, the domain contributed by the TR acts as a reporter molecule and the cellular distribution is determined by the GLUT4 sequence. The power of this approach is that not only can the steady state distribution between the plasma membrane and intracellular compartments be determined, but the individual rates of internalization and externalization (which determine the steady state distribution of membrane proteins) can also be measured. The conclusions of this study are that while the 19-amino acid NH2-terminal domain of GLUT4 promotes internalization of the GTTR chimeras (critically dependent upon the residue at position 5), it does not promote intracellular retention of these chimeras in CHO cells.

Our kinetic analysis is based on the assumption that the chimeras on the cell surface are in equilibrium with those inside the cell. Validating this analysis are the observations that: (a) most (>95%) of the Tf internalized by the chimeras is released from cells with kinetics similar to those observed for cells expressing the wild-type TR; (b) the predicted S/I, based upon the measured initial rates of internalization and externalization, agree with the measured steady state distribution of chimeras between the surface and interior of cells; and (c) intracellular compartments labeled with fluorescent Tf, i.e., accessible to chimeras on the surface, overlap completely with those labeled (in detergent-permeabilized cells) with antibody to the chimeras (indicating the total cellular pool). Taken together, these results demonstrate that the entire intracellular pool of GTTR chimeras is in dynamic equilibrium with the cell surface.

The impetus for these studies was to further define the region of GLUT4 required for maintaining (in the basal state) a predominantly intracellular distribution of GLUT4. The expectation that the amino terminus would serve as an intracellular retention signal was based on the previous findings that this sequence was required for the normal distribution of GLUT4 and that it could confer a GLUT4-like distribution when transferred to another isoform (e.g., GLUT1 which is found predominantly on the cell surface) or to the H1 chain of the asialoglycoprotein receptor (Piper et al., 1992, 1993). In our studies, the distribution of GTTR-F (and to a greater degree, GTTR-Y) between the surface and interior of cells was much like the effectively sequestered chimeras in the aforementioned reports, while GTTR-A displayed a pattern of expression reminiscent of GLUT1 targeting. As stated above, a decrease in ke or a proportionate increase in ki would necessarily affect the steady state distribution of GLUT4 to the same extent. However, it is clear that the unique distribution of each GTTR chimera is due to a modulation of the internalization rate and is not the result of the GLUT4 sequences promoting retention.

Our studies demonstrate that the GLUT4 amino terminus can function as an internalization motif. The observation that the rates of 59Fe accumulation are directly proportional to the internalization rates of the various chimeras demonstrates that the requirements for cellular iron accumulation from internalized Tf are being met, i.e., passage through correct intracellular compartments. Substitution of the GLUT4 amino terminus for that of the TR resulted in a chimeric protein that was endocytosed at half the rate of the wild-type TR. Substitution of tyrosine for phenylalanine at position 5
doubled the internalization rate to a value equal to the wild-type TR, whereas substitution of alanine at position 5 slowed the internalization rate to the rate observed when the cytoplasmic domain of the TR is deleted (i.e., internalization in the absence of a specific internalization motif). The rates of "Fe accumulation by cells expressing the various chimeras confirmed the different internalization rates and attest to the biological activity of the GTTR chimeras. Consistent with our finding that the internalization motif of GLUT4 can be transferred to the TR are the previous observations that the internalization motifs of other receptors (including the low density lipoprotein receptor and the mannose-6-phosphate receptor) were active when transplanted into the cytoplasmic domain of the TR (Collawn et al., 1991).

As previously noted by Piper et al. (1993), the sequence PSGFQQI within the amino-terminal cytoplasmic domain of GLUT4 fulfills the presumed requirements for a functional clathrin-coated pit internalization motif; an aromatic residue separated by two amino acids from a bulky hydrophobic residue, forming a proposed tight turn (Collawn et al., 1990; Kitstakis et al., 1990). All available experimental evidence is consistent with the proposal that GLUT4 undergoes localization within clathrin-coated pits in 3T3-L1 adipocytes (Robinson et al., 1992). Our finding that substitution of an alanine for phenylalanine greatly reduces the internalization of the GTTR chimera is consistent with a previous finding that mutation of phenylalanine to alanine in GLUT4 dramatically reduces the association of GLUT4 with plasma membrane clathrin lattices (Piper et al., 1993). Nuclear magnetic resonance analysis has revealed that the NH2 terminus of GLUT4 forms a tight turn in solution and when an alanine is substituted for phenylalanine the tight turn is not formed (Studelska, D., and D. James, unpublished results). Together, these findings suggest that the PSGFQQI sequence is a functional internalization motif and highlight the importance of the endocytic machinery in the sequestration of GLUT4. The accumulation of glucose transporter isoforms other than GLUT4 on the cell surface appears to be by default while GLUT4’s efficient internalization motif promotes the intravesicular incorporation of a large percentage of this transporter. This proposal opposes the one previously suggested by others (Calderhead et al., 1990; Haney et al., 1991; Hudson et al., 1992) in which non-GLUT4 isoforms are postulated to contain a signal motif which targets them to the cell surface. Interestingly, the native GLUT4 internalization motif (containing a phenylalanine at position 5) provides for an intermediate level of internalization compared to internalization with a tyrosine at position 5. There is precedence for phenylalanine in a less efficient internalization motif within endocytic systems for lysosomal acid phosphatase (Peters et al., 1990), surface glycoproteins (Kitstakis et al., 1990), and mannose-6-phosphate/IGF II (Jadot et al., 1992). The GTTRY chimera with a YYQI motif was internalized with a similar rate to the wild-type TR with its YTRF internalization motif, reinforcing the general observation that tyrosine promotes more efficient internalization than phenylalanine when substituted in identical motifs.

How is the predominant intracellular localization of GLUT4 achieved? Our results demonstrate that the amino terminus of GLUT4 contains a functional internalization motif. It is likely that efficient internalization promoted by this motif accounts for the cellular distributions observed in studies of other chimeric molecules containing the amino terminus of GLUT4 (Piper et al., 1992, 1993). Two recent reports utilizing epitope-tagged GLUT1-GLUT4 chimeras have shown that the carboxy-terminal 30 amino acids of GLUT4 are important for intracellular localization (Czech et al., 1993; Verhey et al., 1993). A view of GLUT4 trafficking consistent with these observations is that both efficient internalization and slowed recycling are required to achieve the steady state intracellular sequestration. This implies that two distinct trafficking motifs are required: the amino-terminal internalization motif and a retention motif (promoting slowed recycling) which is potentially in the carboxy terminus of GLUT4. Maximum sequestration would only be achieved when both motifs are present, underscoring the importance of constitutive, clathrin-coated pit-mediated internalization of GLUT4. It is of interest to note that Verhey et al. (1993) have found that, although the 30 carboxy-terminal amino acids of GLUT4 are primarily responsible for the sequestration of GLUT1-GLUT4 chimeras, the sequences within the amino-terminal third of GLUT4 contribute to the targeting. This observation is consistent with a role for the amino-terminal internalization motif in achieving sequestration. An additional benefit of efficient, constitutive internalization of GLUT4 is that the intracellular retention need not be complete, since any GLUT4 that evades retention would be rapidly returned to an intracellular compartment following internalization from the plasma membrane.

The observations that the majority of native internalization motifs are tyrosine based and that substitution of tyrosine for phenylalanine provides for more rapid internalization of GTTRs raises the question of the possible physiological significance of the intermediate level of internalization directed by the native phenylalanine-based internalization motif. GLUT4 using the phenylalanine-based internalization motif spends approximately twice as much time on the cell surface as it would if it had the more typical tyrosine-based motif (based on the measured internalization rates of the GTTR-F and GTTRY chimeras). Thus, the phenylalanine-based internalization motif may provide a means to maximize surface time during periods of insulin stimulation. It is also possible that a phenylalanine-based but not a tyrosine-based motif may serve some advantage if insulin is found to inhibit GLUT4 internalization (James et al., 1993). Hence, GLUT4 may provide a unique case in preference for a phenylalanine instead of a tyrosine in an aromatic amino acid-based internalization sequence and may provide some hitherto unaddressed clues to the mechanism of insulin-regulated glucose transport.

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