A family of facilitative glucose transporters (GLUTs 1–7) has recently been identified in mammals [1]. It is of key interest to determine the unique function of each GLUT isoform in an effort to broaden our understanding of whole body glucose homeostasis. An isoform of relevance to the regulation of glucose metabolism by insulin is GLUT-4. The expression of this isoform is confined to tissues that exhibit insulin-regulated glucose transport, namely muscle and fat [2,3], and its action is central to the removal of glucose from the blood following the consumption of a meal. Since the discovery of GLUT-4 six years ago our understanding of insulin-regulated glucose transport has increased tremendously. It is now clear that the major mechanism by which insulin acutely stimulates glucose transport is by provoking the movement of GLUT-4 from intracellular vesicles to the cell surface [4].

A prominent feature of GLUT-4, not shared by other glucose transporters, is that in unstimulated cells it is virtually excluded from the cell surface [4]. This property is also demonstrable when GLUT-4 is expressed in heterologous cells, suggesting that the information that specifies the intracellular sequestration of GLUT-4 is encoded in its primary amino acid sequence [5–9]. These types of observations inspired several groups to search for targeting domains within GLUT-4 that potentially mediated its intracellular sequestration. Because other transporter isoforms are constitutively targeted to the cell surface, and in view of the high degree of amino acid sequence similarity among different transporter isoforms, it was reasoned that such an endeavor may be accomplished using a chimeric strategy. Such an approach should allow identification of a domain(s) that is both necessary and sufficient for intracellular sequestration. This approach minimizes non-specific effects due to changes in protein folding and/or the necessity for interactive domains located at different sites within the primary amino acid sequence of the protein.

What types of targeting signals are likely to be found in GLUT-4? Kinetic studies reveal that in both the absence and presence of insulin GLUT-4 undergoes continuous recycling between its intracellular storage depot and the cell surface [10,11]. GLUT-4 is internalized from the cell surface quite efficiently and morphological studies have revealed that GLUT-4 associates with cell-surface clathrin lattices [12]. In the absence of insulin, the externalization rate of GLUT-4 is much slower than that of other recycling receptors, such as the transferrin receptor [13]. Hence, GLUT-4 is likely to contain at least three targeting motifs: an internalization motif, a signal to sort it out of sorting endosomes, and a retention motif to mediate its withdrawal from the recycling pathway.

Substantial progress in identifying sorting signals in a variety of recycling receptors has been made [14]. These studies are encouraging because they indicate that sorting signals are not complex patches nor do they involve the overall three-dimensional structure of the protein. Rather they appear to be made up of linear stretches of amino acids, the critical constituents of which may be as short as 2–4 amino acids. Furthermore, such sorting signals are invariably located within the cytoplasmic tails of many receptors, indicating that they may function by interacting with a cytoplasmic sorting protein(s). Most work has focused on internalization motifs. These studies have revealed that in a wide variety of receptors the presence of an aromatic amino acid, usually a tyrosine, in an appropriate context is sufficient to encode efficient internalization [14]. The transferrin receptor has become the prototype system for studying internalization motifs, mainly because the intracellular itinerary of this receptor is well defined. The sequence Tyr-Thr-Arg-Phe in the cytoplasmic tail of the transferrin receptor encodes a high-efficiency internalization motif. Internalization motifs from other receptors have been shown to replace the high-efficiency internalization function of the Tyr-Thr-Arg-Phe sequence. These aromatic amino-acid-based internalization motifs have been analysed using two-dimensional n.m.r. and have been shown to contain a type II β-turn configuration [14]. Several other studies have shown that this motif functions by binding to adaptins, one of the essential constituents of clathrin-coated vesicles.

Various laboratories have attempted to define targeting domains in GLUT-4 [5–9,15]. Each of these groups have constructed chimeras between GLUT-1, a predominantly cell-surface transporter, and GLUT-4. These constructs have been expressed in a variety of cell types, including
Chinese hamster ovary cells, PC12 cells, NIH3T3 cells, Cos7 cells and Xenopus oocytes. Three groups have independently found that constructs containing the GLUT-4 cytoplasmic C-terminus are localized to an intracellular compartment that resembles the GLUT-4 compartment [5,9,15]. Conversely, constructs containing the GLUT-1 terminus appear to target like GLUT-1. In contrast, Oka and colleagues [6] constructed a series of N-terminal swaps and identified two potential targeting domains in GLUT-4; one around transmembrane domain 3, the putative leucine-zipper domain, and another around transmembrane domains 7 and 8 [6]. However, in this report the converse chimeras were not studied and so it is not clear whether these domains are both necessary and sufficient for GLUT-4 targeting. Finally, my laboratory has reported that the cytoplasmic N-terminus of GLUT-4 is both necessary and sufficient for intracellular sequestration [7,8].

While these reports appear to be quite discrepant it seems likely that there are multiple targeting domains in GLUT-4 and that, for some reason, the role of a particular domain in the overall targeting has been exaggerated in the different systems that have been employed. This conclusion is strengthened by the fact that the targeting motifs in the N- and C-termini of GLUT-4 resemble targeting motifs that have been identified in the cytoplasmic tails of recycling receptors.

We have studied the subcellular distribution of a series of GLUT-4 N-terminal truncations and point mutants in order to define the amino acid constituents of this motif. The essential amino acid motif is Pro-Ser-Gly-Phe-Gln-Gln-Ile, with the phenylalanine playing the most dominant role [7]. This motif resembles an aromatic amino acid-based internalization motif that has been described in a number of recycling receptors as well as in lysosomal membrane proteins. The essential feature of this latter motif is an aromatic amino acid (tyrosine or phenylalanine) and a bulky hydrophobic residue separated from the aromatic by two amino acids. It has also been shown using two-dimensional n.m.r. that these motifs undertake a tight turn structure in solution. We have undertaken a similar analysis of synthetic peptides encompassing the GLUT-4 N-terminus and have found that the Phe-Gln-Gln-Ile motif is located within a type II β turn (D. Studelska and J. Gao, unpublished work).

As mentioned above, aromatic amino acid-based motifs are thought to regulate efficient internalization of cell-surface proteins by facilitating an interaction between the cytoplasmic tail of recycling receptors with adaptins, one of the constituents of clathrin-coated vesicles. In order to determine whether the N-terminus of GLUT-4 could fulfill a similar role we studied the distribution of mutant transporters in purified plasma-membrane fragments attached to glass coverslips [7]. GLUT-4 was enriched in clathrin-coated pits and lattices in the cell surface as determined by double-label immunofluorescence staining of the plasma-membrane fragments with GLUT-4 and clathrin antibodies. In contrast, a mutant transporter in which Phe-5 in GLUT-4 was mutated to alanine showed a large increase in cell-surface staining with no apparent co-localization with cell-surface clathrin.

We have also shown that the GLUT-4 N-terminus mediates intracellular sequestration in the context of a heterologous protein. A hybrid transferrin receptor in which the N-terminal cytoplasmic tail was swapped with that from GLUT-4 was expressed in Chinese hamster ovary cells using stable transfection [16]. These hybrids were found to have a much higher internalization efficiency than tail-less receptors but ~50% of the internalization efficiency of wild-type receptors. Hence, these experiments conclusively show that the N-terminus of GLUT-4 contains a functional aromatic amino acid-based internalization motif.

Studies using the CD3 chain of the T-cell antigen receptor and the mannose-6-phosphate receptor have identified another targeting motif, Leu-Leu, located in the cytoplasmic tails of each of these proteins [17,18]. The GLUT-4 C-terminus, but not that of GLUT-1, contains a similar di-leucine motif at positions 489–490. Birnbaum and colleagues [9] have found that transporters in which these residues are mutated to alanine/serine exhibit a high cell-surface distribution, consistent with this domain playing an important role in intracellular sequestration [9]. The role of this motif in regulating the intracellular sequestration of GLUT-4 remains to be determined. It has been shown that a di-leucine motif mediates efficient internalization in CD3 [18] and when transplanted into the cytoplasmic tail of the transferrin receptor [14]. Thus, Birnbaum and colleagues [17] argued that the di-leucine motif in the GLUT-4 C-terminus may function as an internalization motif. However, the di-leucine motif in the mannose-6-phosphate insulin-like growth factor II (IGF II) receptor does not appear to play a role in regulating internalization [17]. In this protein it was found that the di-leucine motif regulates intracellular sorting of the mannose-6-phosphate/IGF II receptor between endosomes and pre-lysosomes. This is of interest for two reasons.
First, intracellular GLUT-4 vesicles are highly enriched in the mannose-6-phosphate/IGF II receptor [19] and so it is possible that GLUT-4 may follow a similar intracellular trafficking pattern to the mannose-6-phosphate/IGF II receptor. Secondly, the di-leucine motif in both GLUT-4 and the mannose-6-phosphate/IGF II receptor tail is flanked by a consensus casein kinase site. We have previously shown that the serine residue adjacent to the di-leucine motif is the major phosphorylation site in GLUT-4 [20]. Hence, it seems plausible that the di-leucine motif in the GLUT-4 C-terminus may regulate intracellular sorting of the transporter rather than its internalization from the cell surface.

In view of the discrepant results that have been obtained by various laboratories in elucidating targeting motifs in GLUT-4, it will be important to conduct future targeting experiments in a more appropriate cellular context such as adipocytes or muscle cells. Unfortunately, such studies will be complicated by the endogenous expression of GLUT-4 in these cells. This will necessitate epitope-tagging the expressed protein, in which case it will be important to choose a site that has little influence on the normal function of the protein. We have begun investigating this possibility and have expressed a GLUT-4 construct with a foreign epitope inserted as a tag on the C-terminal end of the protein. This construct appears to function indistinguishably from the endogenous GLUT-4 protein and experiments are now underway to examine the distribution of N- and C-terminal mutants in this background. A major question is whether the N- and C-terminal targeting domains function co-operatively in the targeting of GLUT-4. In addition, it will be of interest to determine whether either of these motifs participates in the insulin-regulated movement of GLUT-4. Using adipocytes as the host for transporter mutant expression it should be possible to address these sorts of questions. Finally, it is hoped that once the appropriate targeting domains have been identified it will be possible to elucidate the proteins that interact with these domains. Ultimately, this will provide the critical link between GLUT-4 targeting and the insulin signal transduction pathway.


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