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COMMENTARY

Targeting of mammalian glucose transporters

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INTRODUCTION

Glucose is a basic energy source for most mammalian cells. With the notable exception of luminal epithelial cells in the intestine and kidney, glucose transport occurs by facilitated diffusion. Molecular cloning studies have revealed a family of facilitated glucose transporters, or "GLUTs", the members of which have a high degree of amino acid and structural homology (Fig. 1). Differences in transport kinetics, substrate specificity, tissue expression and subcellular targeting presumably contribute to the metabolic phenotype of a particular cell (Table 1). In this article we will focus on the unique subcellular targeting properties of facilitated glucose transporters because these reveal some of the critical physiological functions of these proteins.

Facilitated glucose transporters provide an ideal system for studying the molecular regulation of membrane protein targeting. In view of the structural similarity between the different isoforms (Fig. 1) it is possible to construct chimeric proteins to identify targeting domains while maintaining the overall structure of the protein (Piper et al., 1992). There is considerable amino acid homology between the different transporters in the membrane spanning domains (Fig. 1), a number of which are thought to form amphipathic α helixes. Hence, this region of the molecule probably forms the hexose pore (Lienhard et al., 1992). The three major cytoplasmic domains exhibit significant divergence in amino acid sequence. These are the domains that likely contain information that is recognized by cytosolic machinery to determine the eventual destination of the protein in the cell. Two separate examples of glucose transporter targeting will be discussed. The first is that of sorting in polarized epithelial cells. The second area, which constitutes the bulk of this article, concerns the regulated sorting of glucose transporters by insulin.

Sorting of glucose transporters in polarized epithelial cells

The sorting of proteins to either the apical or the basal-lateral membrane in an epithelial cell has been a model system for studying the molecular control of protein targeting. Recent studies have begun to define the amino acids in the targeted proteins that determine this sorting specificity (see review by Mostov et al., 1992; Wollner and Nelson, 1992). It is not clear, however, if specific information is required for basal-lateral and apical targeting or if movement to one membrane occurs by default. The recent demonstration that different glucose transporter isoforms are targeted to either cell surface in a polarized epithelial cell may provide a useful system for addressing this question. In Caco-2 cells, a human colonic tumour cell line, GLUT-3 is found entirely within the apical membrane (Harris et al., 1992). This is in contrast to GLUT-1, which is localized to the basal-lateral membrane in Caco2 cells (Harris et al., 1992), and GLUT-2, which is also localized to the basal-lateral membrane in kidney and intestinal epithelial cells (Thorens et al., 1990). The sorting of GLUT-3 is of particular interest because this isoform is normally expressed in parenchymal cells of the brain (Mantych et al., 1992) and is only expressed in epithelial cells as a consequence of cell transformation (Harris et al., 1992; Yamamoto et al., 1990). Hence, its sorting to the apical membrane in epithelial cells may offer insight into its sorting in neurons. It has been proposed that neurons and epithelial cells may utilize the same targeting signals to sort proteins to the apical/axonal membrane versus the basal-lateral/cell body membrane (Dotti et al., 1991). Preliminary support for this hypothesis has been obtained with the observation that in human brain sections GLUT-3 immunolabeling co-localizes with that of an axonal marker (Mantych et al., 1992). By constructing chimeric transporters it should be possible to determine by both loss and gain of function whether there are domains that specifically determine apical and/or basal-lateral targeting.

Insulin-regulated sorting of GLUT-4

GLUT-4 is expressed only in muscle and adipose tissue and is physiologically the most dominant transporter in these tissues (James et al., 1989b). These "insulin-sensitive"

Key words: GLUT, sorting, insulin, metabolism

Table 1. The facilitated glucose transporter family

Isoform	Size (amino acids)	Primary tissue distribution	Function
GLUT-1	492	Brain endothelial cells, erythrocytes, placenta, kidney, colon	Blood/tissue barrier transport, basal transport
GLUT-2	524	Kidney and small intestinal epithelial cells, liver, pancreatic β cells	Low-affinity transporter Basal-lateral transporter in kidney and gut to facilitate glucose entry into blood; hepatic glucose output; part of the glucose sensor in islets and liver
GLUT-3	496	Neurons, placenta	High-affinity transporter Unabated transport into brain parenchymal cells
GLUT-4	509	Skeletal muscle, brown and white adipose tissue, heart	Mediate insulin-regulated glucose transport
GLUT-5	501	Small intestine, kidney, brain endothelial cells, sperm	High-affinity fructose transporter
GLUT-7	528	Liver	Mediate glucose release from the ER coupled to glucose-6-phosphatase

For extensive review of the tissue distribution and function of the facilitated glucose transporters see (Bell et al., 1990). GLUT-6 is not indicated because it has been shown to encode a pseudogene. For details of the cloning and properties of GLUT-7 see (Waddell et al., 1992).

tissues have the capacity to rapidly augment their glucose transport rate in response to insulin and other extrinsic factors, such as exercise, by as much as 10- to 20-fold. This is a result of the rapid movement of GLUT-4 from intracellular membranes to the cell surface. This was first demonstrated using cytochalasin B binding analysis of adipocyte subcellular fractions (Cushman and Wardzala, 1980; Suzuki and Kono, 1980), and subsequently by quantitative immunocytochemistry (Slot et al., 1991b) and cell surface labeling with a novel photoreactive compound (Jhun et al., 1992; Yang et al., 1992). In the basal state GLUT-4 is found almost exclusively within the cell in tubulo-vesicular elements that are clustered either in the *trans*-Golgi reticulum (TGR) or in the cytoplasm, often very close to the cell surface (Slot et al., 1991a,b). Cell surface levels of GLUT-4 are increased by as much as 30-fold with insulin. Co-localization of GLUT-4 with endocytotic markers (Tanner and Lienhard, 1989) and with clathrin-coated lattices and pits in the adipocyte plasma membrane (Robinson et al., 1992; Slot et al., 1991b) indicates that GLUT-4 recycles in both the absence and presence of insulin. Pulse-chase studies using the photoreactive cell surface-labeling compound have confirmed this hypothesis (Jhun et al., 1992; Yang et al., 1992).

The subcellular distribution of GLUT-4 differs from that of GLUT-1. Whereas GLUT-4 is located almost exclusively within the cell in the basal state, a substantial proportion (30-50%, depending upon cell type) of GLUT-1 is found at the plasma membrane under these conditions (Slot et al., 1991a,b). Like GLUT-4, cell surface levels of GLUT-1 are increased by insulin but by a factor of only 2-3 (Piper et al., 1991). It has been proposed that this basic targeting difference distinguishes GLUT-4 as the insulin-regulatable glucose transporter. The difference in targeting between GLUT-1 and GLUT-4 is independent of cell type (Haney et al., 1991; Hudson et al., 1992; Shibasaki et al., 1992). While factors such as expression levels of transporter per

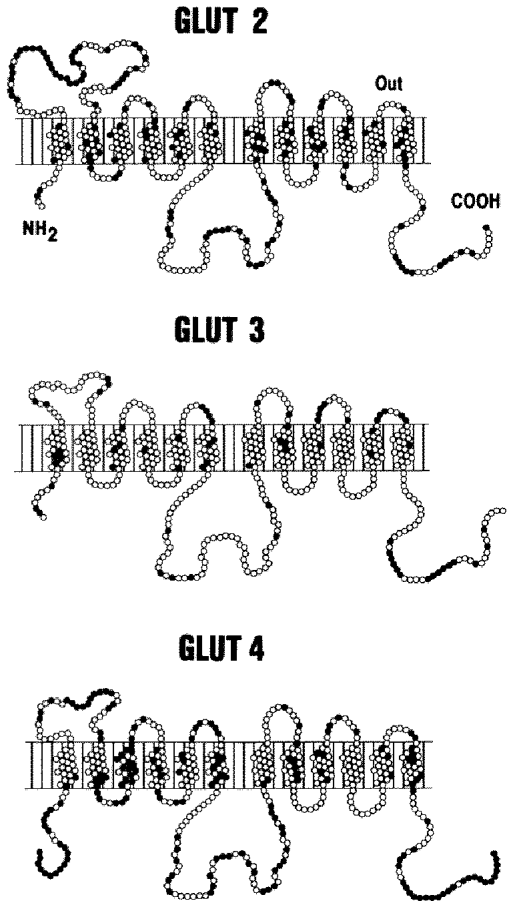

Fig. 1. Heterology plot of glucose transporter isoforms. Unique amino acids specific to GLUT-2, GLUT-3 or GLUT-4 are shown in black. These were determined by eliminating amino acids (shown as circles) that were either not conserved across species for a given GLUT or that were conserved between individual isoforms (GLUTs 1-4). The transmembrane region is indicated by the cross-hatched box. Both the amino and carboxy termini of each isoform are exposed to the cytoplasm. These isoform-specific domains are likely candidates for the targeting specificity of the different isoforms.

cell or cell density may modify transporter subcellular distribution, clearly the primary determinant is the transporter itself. GLUT-4 has been transfected into a variety of "insulin-resistant" cells (i.e. cells that do not exhibit a marked increase in glucose transport in response to insulin) and in each case it is found predominantly in intracellular tubulo-vesicular structures. Intriguingly, the intracellular distribution of GLUT-4 in a transfected fibroblast is similar to that observed in "insulin-sensitive" cells (Haney et al., 1991). Despite this, insulin does not alter the subcellular distribution of GLUT-4 in fibroblasts (Hudson et al., 1992; Robinson et al., 1992; Shibasaki et al., 1992).

The amino terminus of GLUT-4 contains targeting information

Two questions concerning GLUT-4 targeting emerge from the above studies. First, is there a targeting motif in the GLUT-4 sequence that accounts for its efficient intracellular sequestration? Second, are there additional domains within GLUT-4 that provide the basis for its marked translocation to the plasma membrane in response to insulin? We have addressed the first question by analyzing the targeting of chimeric glucose transporters comprising portions of GLUT-4 and GLUT-1 (Piper et al., 1992). In order to avoid the potential complication of a translocation-specific domain these studies were performed in insulin-insensitive Chinese hamster ovary (CHO) cells. These studies indicated that within the context of a multi-spanning glucose transporter the N-terminal cytoplasmic tail of GLUT-4 is both necessary and sufficient for intracellular targeting. This region can also direct the efficient sequestration of a heterologous bitopic membrane protein clearly defining this region of GLUT-4 as an autonomous sorting domain (unpublished data). This finding is consistent with the predictions outlined in Fig. 1, which show that the N terminus of GLUT-4 is unique compared to other transporter isoforms but is conserved among mouse, rat and man. Interestingly, this domain is encoded by its own exon (Bell et al., 1990), which presumably facilitated this targeting adaptation during the evolution of the glucose transporter family.

Further dissection of the GLUT-4 N terminus has revealed that it shares homology with endocytic signals in cell surface receptors (Fig. 2). Deletional analysis indicates that the targeting information is located within the first 8 amino acids (unpublished data). Alanine scanning mutagenesis has pinpointed Pro2, Ser3, Phe5 and Ile8 as important amino acids within this domain, with Phe5 playing a critical role (unpublished data). The essential features of putative internalization motifs in other proteins (Fig. 2) is the presence of a Tyr at position 1 and a bulky hydrophobic amino acid at position 4. In some cases Phe can substitute for Tyr at position 1 without loss of function. Therefore, the sequence FQQI within the GLUT-4 N-terminal tail broadly fits the consensus for an internalization sequence. This is consistent with the fact that GLUT-4 constantly recycles (Jhun et al., 1992; Yang et al., 1992) and that it is localized to clathrin lattices at the cell surface (Lienhard, 1989; Piper et al., 1991; Robinson et al., 1992; Slot et al., 1991b).

Our findings implicating the N terminus of GLUT-4 as being an important targeting domain have recently been contradicted (Asano et al., 1992). In these studies four different GLUT-1/GLUT-4 chimeras were expressed in CHO cells to evaluate loss of the specific GLUT-4 targeting phenotype. Here it was reported that neither the amino nor the carboxy terminus of GLUT-4 contained essential targeting information. The major difference between the two studies was that Asano et al. (1992) used a stable expression system, whereas in our studies (Piper et al., 1992) a transient system was used, to produce recombinant protein. One possibility is that the transient expression system favours use of the N-terminal internalization motif for targeting whereas the stably produced transporter may rely on an alternate targeting domain. Asano et al. (1992) have localized the targeting domain to around transmembrane domains 7 and 8, a region that is highly conserved among the different transporter isoforms (Fig. 1). Therefore, it is possible that this region has a conserved function such as regulation of transporter oligomerization. It will be imperative to determine if this alternate targeting domain can function both in the context of GLUT-1 and/or in a heterologous protein in order to prove that it is a *bona fide* targeting motif. However, the ultimate test will be to determine the physiological role of these domains by expressing heterologous proteins in insulin-sensitive cells.

Nevertheless, the fact that the N-terminal targeting motif in GLUT-4 resembles a well-characterized targeting motif supports our conclusions and lends itself to speculation that the exclusion of GLUT-4 from the cell surface in the basal state is mediated entirely by rapid endocytosis of GLUT-4 from the cell surface. However, by analogy with other recycling proteins we tend to think this is not the case. As noted above, GLUT-4 is predominantly (>90%) intracellular under basal conditions, irrespective of cell type. In contrast, as much as 40% of the total transferrin receptor population, a protein that has a very efficient internalization signal, is present at the plasma membrane in fibroblasts (Jing et al., 1990). Furthermore, the intracellular distribution of GLUT-4 in adipocytes is distinct from GLUT-1 (Piper et al., 1991) or the transferrin receptor (D. Hanpeter and D. E. James, unpublished data). If the cellular locations of these proteins were regulated entirely by their relative rates of endocytosis one would expect a random distribution of these proteins throughout recycling endosomes. The fact that this is not the case raises the possibility that the intracellular sequestration of GLUT-4 may be due at least in part to intracellular retention, a property not observed for the transferrin receptor (Jing et al., 1990). This is supported by the relatively low externalization rate constant of GLUT-4 (Jhun et al., 1992) compared to other proteins, such as the transferrin receptor (Tanner and Lienhard, 1987), in adipocytes. It will be of interest to determine whether this additional sorting information is contained within the N-terminal tail of GLUT-4.

We propose, therefore, that the effective exclusion of GLUT-4 from the cell surface in a non-stimulated insulin-sensitive cell is due to retention in intracellular vesicles and to efficient internalization of GLUT-4 from the cell surface via the clathrin-mediated pathway. We envisage that both of these processes are regulated by the binding of targeting machinery to cytoplasmic domains of GLUT-4. The N

terminus of GLUT-4 is clearly a good candidate for regulating the internalization rate of GLUT-4. However, it remains to be determined if this or other domains, such as that proposed by Asano et al. (1992), mediates the intracellular retention of GLUT-4. It is relevant that aromatic amino acids in the appropriate context of cytoplasmic tails have been shown to regulate both internalization (Canfield et al., 1991; Collawn et al., 1990; Davis et al., 1987; Fuhrer et al., 1991; Jing et al., 1990; Peters et al., 1990) and retention (Roberts et al., 1992) for a number of different proteins. Thus, it is conceivable that the retention mechanism for proteins in the endosomal pathway is similar to the process by which cell surface molecules are specifically packaged into clathrin-coated pits (Pearse and Robinson,1990). This being the case one would expect conservation in both the targeting machinery and the signals recognized by this machinery for regulating both processes.

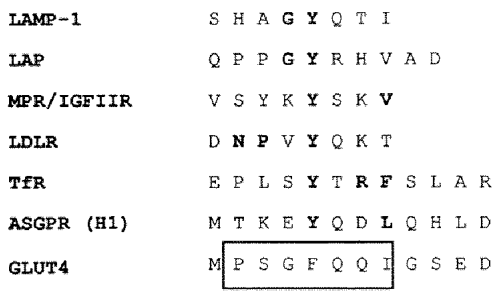

Fig. 2. Cytoplasmic domains that are involved in targeting in the endocytic/lysosomal system compared with the putative sequestration domain of GLUT-4. Regions of significance are alligned in the amino- to carboxy-terminal direction. Amino acids that are critical are in bold, or boxed in the case of GLUT-4. Targeting domains are shown for: two lysosomal type I membrane proteins-LAMP-1 (see review by Fukuda, 1991) and lysosomal acid phosphatase (LAP) (Peters et al., 1990); two type I membrane proteins that undergo efficient endocytosis: mannose 6-phosphate/IGF II receptor (MPR/IGF IIR) (Canfield et al., 1991) and the low density lipoprotein receptor (LDLR) (Davis et al., 1987); two type II membrane proteins that are efficiently endocytosed: transferrin receptor (TfR) (Collawn et al., 1990) and the H1 subunit of the asialoglycoprotein receptor (ASGPR) (Fuhrer et al., 1991).

Insulin increases movement through the recycling pathway - Is GLUT-4 unique?

It is unclear from the above studies if the N-terminal sequestration domain of GLUT-4 plays a direct role in insulin-dependent movement of the protein to the cell surface. This issue is complicated by the fact that many recycling proteins (e.g. the transferrin receptor, mannose 6-phosphate receptor, and α2-macroglobulin receptor) are also translocated to the cell surface upon insulin stimulation (see review by Lienhard, 1989). Hence, it is possible, as suggested previously (Lienhard, 1989), that insulin simply accelerates bulk movement through the exocytic pathway, resulting in an increase in the steady-state distribution of many recycling proteins, including GLUT-4, at the cell surface. However, in contrast to the 20- to 30-fold increase in cell surface levels of GLUT-4 (Slot et al., 1991b), insulin causes only a 2- to 3-fold increase in cell surface levels of other proteins (Lienhard, 1989). Establishing the basis for this difference will be critical in understanding the function and regulation of GLUT-4 in insulin-sensitive cells. Recent kinetic studies of GLUT-4 recycling in adipocytes, using an impermeant photoreactive glucose analogue, have provided a major clue (Jhun et al., 1992). Insulin increased the GLUT-4 exocytic rate constant by 3.3-fold and decreased the internalization rate constant by 2.8-fold. In contrast, insulin increased the exocytic rate constant for the transferrin receptor (Tanner and Lienhard, 1987) and the mannose 6-phosphate receptor (Oka et al., 1985) by approximately 2-fold in adipocytes without altering the internalization rate constant. Thus, the additional effect of insulin to slow the endocytosis of GLUT-4 may distinguish the insulin-dependent translocation of GLUT-4 from that of other proteins such as the transferrin receptor and the mannose 6-phosphate receptor. It is especially interesting that the putative internalization domain of GLUT-4 is of potentially lower affinity compared with homologous motifs in the mannose 6-phosphate receptor or the transferrin receptor (Fig. 2). This may provide GLUT-4 itself with the necessary adaptability to modify its internalization efficiency from high to low in response to insulin. This could be mediated by direct modification of the transporter, perhaps by phosphorylation (James et al., 1989a), or a change in its oligomeric state (Jacobs et al., 1987). Alternatively, insulin may modify a yet uncharacterized GLUT-4 chaperone protein or the sorting machinery itself (e.g. phosphorylation of adaptins or clathrin). These modifications may alter the affinity of the internalization regulatory machinery for the GLUT-4 motif without changing the interaction between this machinery and other receptor tails. In support of this notion, we have shown that insulin decreases the percentage of surface GLUT-4 associated with clathrin lattices and pits (Robinson et al., 1992), implying that insulin may inhibit entry of GLUT-4 into clathrin-coated pits.

Insulin-stimulated translocation: the missing link

The fact that GLUT-4 is unable to undergo insulin-stimulated translocation to the cell surface when expressed in a fibroblast (Hudson et al., 1992; Robinson et al., 1992; Shibasaki et al., 1992) underscores the complexity of what is clearly a cell-specific process. This observation implies that essential factors are either missing from fibroblasts or that fibroblasts express a dominant inhibitory factor that is not present in insulin-sensitive cells. For the reasons described below, we favour the view that essential factors are missing from fibroblasts.

GLUT-4 appears to be targeted to specialized intracellular vesicles in fat and muscle cells that resemble regulated exocytic vesicles. Therefore, the basis for the remarkable regulation of GLUT-4 by insulin may reflect the biogenesis of this specific organelle. In adipocytes GLUT-4 has been co-localized with a synaptic vesicle protein, synaptobrevin (Cain et al., 1992). Adipocytes also express a small molecular weight GTPase called Rab3D (Baldini et al., 1992), which is highly homologous to the synaptic vesicle GTPase Rab3A. It has been proposed that the Rab3 family of GTPases may be involved in regulated exocytosis and so it is distinctly possible that Rab3D is in some way

involved in regulating GLUT-4 exocytosis. We have recently been able to isolate biochemically a subpopulation of GLUT-4 vesicles from 3T3-L1 adipocytes that are highly insulin-regulatable and enriched in GLUT-4 but depleted of endocytic markers (Robinson and James,1992). Furthermore, the polypeptide composition of these vesicles isolated from different insulin-sensitive cells is well conserved (D. Hanpeter, D. E. James, unpublished data).

An alternate explanation for the cell specificity of GLUT-4 movement is that GLUT-4 is targeted appropriately in fibroblasts but these cells lack the appropriate signal transduction machinery to trigger insulin-dependent translocation. The insulin receptor does not appear to be the limiting factor because GLUT-4 does not move in CHO cells that overexpress the receptor (Hudson et al., 1992). Another candidate is the IRS-1 protein. This protein is one of the first to be phosphorylated on tyrosine following insulin stimulation (Backer et al., 1992), it binds putative effector molecules such as PI-3-kinase in an insulin-sensitive fashion (Backer et al., 1992) and its level of expression is increased considerably following differentiation of 3T3-L1 fibroblasts into insulin-sensitive adipocytes (Rice et al., 1992). The involvement of cell-specific GTP binding proteins or their effectors is implicated by the observation that GTPγS stimulates translocation of GLUT-4 in adipocytes but not in fibroblasts (Baldini et al., 1991; Robinson et al., 1992). Finding this missing link would greatly increase our understanding of insulin action and the control of glucose metabolism.

Conclusion

The glucose transporter family of proteins may prove to be an ideal system for studying the molecular regulation of protein targeting in the cell. The virtue of a chimeric analysis for identifying putative ligand-specific targeting domains is that an assessment of their necessity and sufficiency can be made. Such an analysis overcomes many of the problems encountered using other approaches. Using this approach to ascertain why the insulin-regulatable transporter GLUT-4 is so efficiently sequestered within the cell in the absence of insulin we have identified a domain that is homologous to tyrosine-containing endocytosis signals. This domain may also be implicated in the insulin-dependent sorting of the protein because recent studies indicate that the internalization rate of GLUT-4 in adipocytes may be inhibited by insulin. In the future it will be important to identify the machinery that recognizes and sorts this domain in an effort to understand precisely how insulin regulates the subcellular distribution of this protein so exquisitely.

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