The cytosolic C-terminus of the glucose transporter GLUT4 contains an acidic cluster endosomal targeting motif distal to the dileucine signal

Annette M. SHEWAN*[1], Brad J. MARSH*[1,2], Derek R. MELVIN†, Sally MARTIN*, Gwyn W. GOULD† and David E. JAMES*[3]

*Institute for Molecular Bioscience and the Department of Physiology and Pharmacology, University of Queensland, St. Lucia, Queensland 4072, Australia, and †Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Davidson Building, Glasgow G12 8QQ, Scotland, U.K.

The insulin-responsive glucose transporter GLUT4 is targeted to a post-endocytic compartment in adipocytes, from where it moves to the cell surface in response to insulin. Previous studies have identified two cytosolic targeting motifs that regulate the intracellular sequestration of this protein: FQQI[5–8] in the N-terminus and LL[489,490] (one-letter amino acid notation) in the C-terminus. In the present study we show that a GLUT4 chimaera in which the C-terminal 12 amino acids in GLUT4 have been replaced with the same region from human GLUT3 is constitutively targeted to the plasma membrane when expressed in 3T3-L1 adipocytes. To further dissect this domain it was divided into three regions, each of which was mutated *en bloc* to alanine residues. Analysis of these constructs revealed that the targeting information is contained within the residues TELEYLGP[498–505]. Using the transferrin–horseradish peroxidase endosomal ablation technique in 3T3-L1 adipocytes, we show that mutants in which this C-terminal domain has been disrupted are more sensitive to chemical ablation than wild-type GLUT4. These data indicate that GLUT4 contains a targeting signal in its C-terminus, distal to the dileucine motif, that regulates its sorting into a post-endosomal compartment. Similar membrane-distal, acidic-cluster-based motifs are found in the cytosolic tails of the insulin-responsive aminopeptidase IRAP (insulin-regulated aminopeptidase) and the proprotein convertase PC6B, indicating that this type of motif may play an important role in the endosomal sequestration of a number of different proteins.

Key words: adipocyte, endosome, insulin, trafficking, translocation.

INTRODUCTION

Specialized compartments related to the endosomal–lysosomal system that give rise to cell-specific functions, such as synaptic-vesicle exocytosis and antigen presentation, have been described in a variety of cell types [1]. The insulin-regulated movement of GLUT4 to the cell surface in myocytes and adipocytes may provide another example of this type of regulated recycling. In the resting state, GLUT4 is localized to tubulo-vesicular elements that are clustered either in the *trans*-Golgi region, endosomes or in the cytoplasm [2,3]. The extremely low levels of GLUT4 at the plasma membrane under these conditions appears to be a crucial feature of this protein that distinguishes it from other glucose-transporter-protein isoforms, such as GLUT1. Despite marked differences in the cell-surface levels of GLUT4 and GLUT1 in unstimulated 3T3-L1 adipocytes, their endocytic rates are essentially identical [4]. Therefore it is unlikely that the exclusion of GLUT4 from the plasma membrane in basal adipocytes results solely from its efficient internalization. Consequently, it has been proposed that GLUT4 is targeted to a unique intracellular compartment(s) in differentiated muscle and fat cells that not only facilitates its storage, but also its exocytosis. This enables GLUT4 to move transiently to the cell surface in response to extracellular stimuli such as insulin [2,3,5].

The presence of discrete sorting signals in the cytosolic tails of membrane proteins such as GLUT4 regulates their differential distribution within the endosomal–lysosomal system. In many cases these signals bind to coat components that are thought to selectively transport molecules from one compartment to another [6,7]. Two major types of signals have been described that are identified either by the sequence YXXØ (where Y is an aromatic amino acid, X is any amino acid and Ø is an amino acid with a bulky hydrophobic group) or LL (where L is leucine or isoleucine) [8,9]. Both of these motifs bind to adaptor protein (AP)-1 and AP-2 adaptor complexes that regulate clathrin assembly at the *trans*-Golgi network (TGN) or the cell-surface respectively, and to the AP-3 adaptor complex that has been implicated in endosomal–lysosomal sorting [7,10]. These signals thereby facilitate the efficient delivery of both newly synthesized and internalized proteins to the endosomal system, from where they may either be recycled or transported to the lysosome [8,9]. Thus the presence of multiple and distinct sorting signals within the cytosolic tails of proteins with complex trafficking itineraries is probably required to facilitate their interaction with different adaptor-protein subunits at multiple sites throughout the cell. Certain motifs, such as those found in the cell-surface proteoglycans CD3γ and CD4 and the insulin-like growth factor II/mannose 6-phosphate receptor (IGFII/MPR), have been shown to bind more avidly to AP-1 than to AP-2, which correlates with their roles in sorting at the TGN [11,12]. In contrast, other signals appear to preferentially bind AP-2, suggesting that these motifs predominantly regulate internalization from the cell

Abbreviations used: AP, adaptor protein; IGFII/MPR, the insulin-like growth factor II/mannose 6-phosphate receptor; FCS, foetal calf serum; TGN, *trans*-Golgi network; PM, plasma membrane; LDM, low-density microsomes; HDM, high-density microsomes; M/N, mitochondria/nuclei; HRP, horseradish peroxidase; Tf, transferrin; DMEM, Dulbecco's modified Eagle's medium; IRAP, insulin-regulated aminopeptidase; HA, influenza haemagglutinin epitope.

[1] Both authors contributed equally to this work.

[2] Present address: Boulder Laboratory for 3-D Fine Structure, Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, CO 80309-0347, U.S.A.

[3] To whom correspondence should be addressed (e-mail D.James@cmcb.uq.edu.au).

surface [8,13,14]. In addition, dileucine-based motifs in the cytosolic tails of the lysosomal membrane protein LIMP-II and the melanosome-associated protein tyrosinase selectively bind AP-3, rather than AP-1 or AP-2 [15]. The differential affinity of targeting signals for discrete adaptor subunits may be further influenced by amino acids either proximal or distal to the primary signal [15–17].

Several laboratories have attempted to identify targeting motifs in GLUT4 by examining the subcellular distribution of either chimaeras comprised of different portions of GLUT4 and another glucose transporter isoform, GLUT1, or GLUT4 point mutants [18–20]. Two distinct motifs have been identified: a phenylalanine-based motif ($FQQI^{5-8}$) in the cytosolic N-terminus and a dileucine motif ($LL^{489,490}$) in the cytosolic C-terminus [21–23]. These signals function autonomously as internalization motifs [22,24–27], and so presumably interact with AP-2 at the cell surface. However, these motifs also regulate alternative sorting functions, such as those related to the intracellular sequestration of GLUT4 [28].

It has been suggested that the C-terminus of endogenous GLUT4 contains additional targeting information, based on analyses of chimaeric transporter proteins expressed in insulin-responsive cells [27,29]. In the present study we found that replacing the last 12 amino acids in GLUT4 with those from GLUT3 caused its constitutive accumulation at the cell surface. Addition of the same 12 residues to full-length GLUT4 did not impair targeting, suggesting that the extreme C-terminus of GLUT4 may contain important targeting information. To further dissect this domain, we constructed three separate mutants in which the residues TELE, YLGP or DEND were mutated to AAAA. Analysis of these constructs by subcellular fractionation and endosomal ablation revealed an important role for the residues $TELEYLGP^{498-505}$ in determining the steady-state distribution of this protein in 3T3-L1 adipocytes.

EXPERIMENTAL

Cell culture

3T3-L1 murine fibroblasts obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) bovine-calf serum (CSL Limited, Parkville, Victoria, Australia). Fibroblasts were induced to differentiate 1 day after reaching confluence by the addition of DMEM containing 10% (v/v) heat-inactivated foetal-calf serum (FCS; Gibco BRL), 4 μg/ml insulin, 0.25 mM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine and 100 ng/ml *d*-biotin. After 72 h, the differentiation medium was replaced with fresh FCS/DMEM containing 4 μg/ml insulin and 100 ng/ml *d*-biotin. Adipocytes were re-fed with FCS/DMEM every 72 h, and utilized for experiments at least 10 days following the initiation of differentiation.

Antibodies

The anti-peptide polyclonal antibodies specific for either the 12 C-terminal residues of GLUT4, the 12 or 14 C-terminal residues of human GLUT3 or the 15 N-terminal residues of GLUT4 have been characterized and described elsewhere [30,31]. The affinity-purified polyclonal rabbit antiserum generated against the cytosolic domain of the insulin-regulated aminopeptidase (IRAP) vp165 was generously provided by Dr. Susanna R. Keller, Department of Biochemistry, Dartmouth Medical School, Hanover, NH, U.S.A. The monoclonal anti-(influenza haemagglutinin epitope) (anti-HA) antibody (16B12) was obtained from BabCO, Richmond, CA, U.S.A.

Construction of recombinant GLUT4 transporter cDNAs

The C-terminal GLUT4 mutants were generated by PCR as described previously [32]. *Bgl*II–*Xho*I fragments comprising the C-terminus of GLUT4 and the human GLUT3 epitope tag were generated by PCR and subcloned into the pIRGT backbone [22,32] to generate pTAG (full-length GLUT4 with the addition of the 12 C-terminal amino acids from human GLUT3 at the extreme C-terminus), pTAIL (in which the GLUT4 cDNA sequence coding for the C-terminal 12 amino acid residues was replaced with the corresponding sequence from human GLUT3), pTELE (in which the residues $TELE^{498-501}$ were mutated to alanine residues), pYLGP (in which the residues $YLGP^{502-505}$ were mutated to alanine residues), and pDEND (in which the residues $DEND^{506-509}$ were mutated to alanine residues).

All regions of the cDNAs generated by PCR were completely sequenced. GLUT4 constructs were initially subcloned as *Xba*I–*Xho*I fragments into a shuttle vector that was essentially pBluescript with a modified multiple cloning site, and were then removed and inserted directionally into the unique *Bam*HI–*Kpn*I or *Bam*HI–*Eco*RI sites of the mammalian expression vector pMEXneo (kindly provided by Dr. Eugenio Santos, National Institutes of Health, Bethesda, MD, U.S.A.).

An exofacial HA epitope-tagged, human GLUT4 construct, pCIS2/HA-GLUT4 [33] (kindly provided by Dr. Michael Quon, National Institutes of Health, Bethesda, MD, U.S.A.), was used to generate pHA-GLUT4. Briefly, an *Xba*I fragment containing HA-GLUT4 was excised from pCIS2/HA-GLUT4 and subcloned into pSU21. Flanking *Bam*HI and *Sal*I sites were then used to shuttle the HA-GLUT4 insert into the corresponding sites of the retroviral expression vector pBabepuro [34], to form pHA-GLUT4.

The TAIL, ELEY and YLGP mutations were also generated in pHA-GLUT4. The resulting constructs are referred to as pHA-TAIL, pHA-ELEY and pHA-YLGP respectively. To generate these mutants we took advantage of a unique and conserved *Sac*I site present in the human and rat GLUT4 cDNAs. In the case of pHA-TAIL, a *Bam*HI–*Sac*I fragment from pSU21/HA-GLUT4, encompassing the N-terminal two-thirds of HA-tagged human GLUT4, was first ligated to a *Sac*I–*Eco*RI fragment, encompassing the corresponding rat GLUT4 C-terminus from pTAIL and shuttled into pBSSK. Subsequently, this fragment was excised and subcloned into pBabepuro. A similar strategy was used to construct pHA-ELEY, except that PCR was employed utilizing the primers – 5′-ATCGTGGCCATATTTG-GC-3′ and 5′-CGGAATTCTCAGTCATTCTCATCTGGCCC-TAAGGCTGCAGCTGCTGTACTGGGTTTCAC-3′ – to generate a *Sac*I–*Eco*RI fragment encompassing the rat GLUT4 C-terminus and containing the 499–502 alanine substitutions. Similar to pHA-ELEY, pHA-YLGP was constructed utilizing the PCR primers – 5′-GGCCGGACATTTGACCAGATCT-CG-3′ and 5′-CGGGGATCCTCAGTCATTCTCATCTGCC-GCTGCGGCTTCAAGTTCTGTACTGGG-3′ – to generate a *Bgl*II–*Bam*HI fragment encompassing the 502–505 alanine substitutions within the rat GLUT4 C-terminus. The integrity of all cDNA fragments generated by PCR were confirmed by sequencing.

Expression of GLUT4 mutants in 3T3-L1 adipocytes

cDNA constructs subcloned into the pMEXneo vector were transfected into subconfluent 3T3-L1 fibroblasts using

the LipofectAMINE reagent, following the manufacturer's protocol (Gibco BRL). Neomycin-resistant colonies (0.8 mg/ml G418) (Gibco BRL) were isolated and selected as described previously [32].

Retroviral stocks of HA-GLUT4, HA-TAIL, HA-ELEY and HA-YLGP were generated using the BOSC 23 packaging cell line [35]. To generate 3T3-L1 adipocytes stably expressing each construct, 3T3-L1 fibroblasts (plated at a density of 5×10^5/100 mm plate 16 h beforehand) were infected with the relevant virus for 3–5 h in the presence of 4 μg/ml Polybrene (Sigma). After a 48 h recovery period, infected cells were then selected in DMEM containing 10 % FCS and supplemented with 2 μg/ml puromycin (Sigma). Typically, 40–50 % of cells survived this treatment. These polyclonal pools of 3T3-L1 fibroblasts were then grown to confluence and differentiated as described above. Puromycin was not included in the differentiation medium, but was re-applied once the differentiation regime was completed.

Differential centrifugation

Subcellular membrane fractions were prepared from basal and insulin-treated adipocytes by differential centrifugation using a protocol previously described in detail [32,36]. This protocol yields four membrane fractions designated as high-density microsomes (HDM), low-density microsomes (LDM), plasma membranes (PM) and mitochondria/nuclei (M/N). Here we have focused on the PM and LDM fractions because these fractions are enriched in cell-surface markers and membranes encompassing intracellular GLUT4 respectively [36].

Preparation and use of horseradish peroxidase (HRP)-conjugated transferrin (Tf)

The Tf–HRP conjugate was prepared and used exactly as described previously [37]. Cells were used for ablation experiments 8–12 days post-differentiation. Human apo-transferrin and all reagents for Tf–HRP synthesis were from Sigma. ^{125}I-labelled transferrin and ^{125}I -labelled goat anti-rabbit antibody were from DuPont/NEN.

Electrophoresis and immunoblotting

Total cell membranes or subcellular membrane fractions (10 μg of total protein) were subjected to SDS/PAGE using 7.5 % or 10 % polyacrylamide resolving gels. The protein concentrations of membrane fractions were determined using the bicinchoninic acid ('BCA') assay (Pierce). Proteins were electrophoretically transferred to PVDF transfer membranes (Millipore) and immunoblotted with polyclonal or monoclonal antibodies as indicated. Primary antibodies were detected by probing with either HRP-conjugated donkey anti-rabbit or sheep anti-mouse secondary antibodies and ECL® (Amersham) or SuperSignal (an ECL alternative from Pierce) or ^{125}I-labelled Protein A (Amersham). Autoradiograms were quantified using a model GS-670 imaging densitometer (Bio-Rad). Chemiluminescent bands were quantified directly using a Lumi-Imager F1 (Boehringer Mannheim), and ^{125}I-labelled Protein A blots were quantified directly using a model GS-363 molecular-imaging system (Bio-Rad). The level of GLUT4 (wild-type or recombinant) or IRAP at the PM of insulin-treated adipocytes was nominally assigned a value of 1 to normalize between independent experiments and between recombinant GLUT4 constructs expressed by different cell lines.

RESULTS

Expression of recombinant GLUT4 proteins in 3T3-L1 adipocytes

In the present studies we have used two different strategies to dissect a new targeting domain in the extreme C-terminus of GLUT4. Our initial studies are based on a strategy previously employed by our laboratory where we engineered an epitope, based on the last 12 amino acids of human GLUT3, at the extreme C-terminus of GLUT4, to allow us to discriminate between recombinant and endogenous GLUT4 in stably transfected 3T3-L1 adipocytes [32]. We have previously found that the targeting of this reporter protein, referred to as 'TAG', is indistinguishable from endogenous GLUT4 in multiple clonal cell lines and over a wide range of expression levels [28,32,38]. In the present study we have constructed a variety of mutants within TAG in which various residues within the last 12 amino acids of GLUT4 have been mutated to alanine. In addition, we have constructed an additional construct, referred to as 'TAIL', in which the C-terminal 12 amino acids of GLUT4 (TELEYLGPDEND) were entirely replaced with the corresponding sequence from human GLUT3 (SIEPAKETTTNV). We have studied the steady-state distribution of these constructs in both the absence and presence of insulin in a number of different stably transfected cell lines. To address potential problems associated with the use of stable cell lines we devised a separate strategy in which we employed a high-titre retroviral expression system that allowed us to generate polyclonal pools of 3T3-L1 adipocytes expressing each construct at levels comparable with those of endogenous GLUT4 [34]. In the latter strategy, the same parental 3T3-L1 cell line was infected with high-titre retroviruses expressing the different C-terminal mutants; analyses of the steady-state distributions of all the different retrovirally expressed constructs were then performed in parallel. As an internal control for the integrity of the cell lines following transfection/infection and differentiation in culture, as well as the fidelity of the fractionation protocol, we also analysed the distribution of IRAP, a protein that is targeted similarly to GLUT4 in adipocytes (Figures 1, 2 and 3) [39].

Analysis of stable 3T3-L1 cell lines expressing C-terminal GLUT4 mutants

When expressed in adipocytes, all of the mutants under investigation generated protein products in the range of 40–45 kDa, similar to endogenous GLUT4 (Figures 1 and 2). Total membranes prepared from each cell line were immunoblotted with antibodies specific for either the C-terminus of GLUT4, the GLUT3 epitope tag (to quantify the relative levels of expression of recombinant GLUT4) or the N-terminus of GLUT4 (to assess the total level of recombinant plus endogenous GLUT4 expression). The polyclonal antiserum specific for the GLUT4 C-terminus used in these studies was generated against a synthetic peptide corresponding to residues 498–509 [30] and hence did not recognize TAIL (Figure 1). The epitopes appeared to be partially disrupted in each of the C-terminal alanine mutants (results not shown). Analyses were performed in several different 3T3-L1 cell lines that were selected on the basis of the expression level of the recombinant protein. Cell lines that expressed recombinant GLUT4 at levels comparable with, or lower than, those of endogenous GLUT4 were designated 'low expressors', whereas those expressing at levels $\geqslant$ 2-fold higher than endogenous GLUT4 were designated 'high expressors'. We have previously found that the targeting of TAG is indistinguishable from that of endogenous GLUT4, even at levels that are 4–6-fold higher than endogenous expression levels [28,32,38].

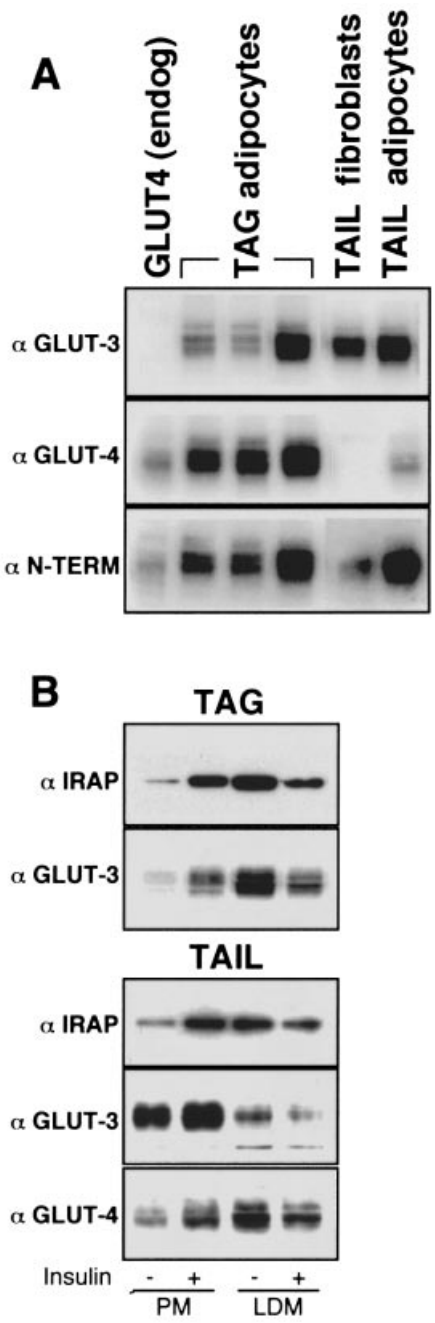

Figure 1 Subcellular distribution of epitope-tagged wild-type GLUT4 (TAG) and a C-terminal chimera (TAIL) in 3T3-L1 adipocytes

(**A**) Expression level of recombinant GLUT4 mutants in 3T3-L1 adipocytes and fibroblasts compared with endogenous GLUT4 ['GLUT4 (endog)']. Total cell membranes prepared from individual cell lines were subjected to SDS/PAGE and immunoblotted with antibodies specific for the GLUT3 epitope, the C-terminus of GLUT4 or the N-terminus of GLUT4. (**B**) Representative immunoblots showing the subcellular distribution of TAG, TAIL and endogenous IRAP and GLUT4 in 3T3-L1 adipocytes incubated in the absence (−) or presence (+) of insulin. PM or LDM were obtained by differential centrifugation. The distribution of TAG and TAIL were determined using the anti-GLUT3 antibody. The TAG and TAIL clones used for these studies expressed the recombinant protein at similar levels.

Table 1 PM/LDM ratios of endogenous and recombinant GLUT4 and endogenous Vp165 in basal 3T3-L1 adipocytes

Adipocytes were subjected to subcellular fractionation by differential centrifugation to obtain fractions enriched either in PM or the intracellular GLUT4 compartment (LDM). The PM/LDM ratios, which provide an index of the intracellular sequestration of transporters in the absence of insulin stimulation, were calculated from multiple independent experiments involving immunoblotting with antibodies specific for either Vp165, the human GLUT3 epitope, the HA epitope or the C-terminus of GLUT4. Clones were classified into two broad categories as described in the Results section (low and high) based upon the level of expression of recombinant GLUT4 relative to endogenous levels. Values are expressed as means ± S.E.M. (arbitrary units/μg of protein), where the number of independent observations (n) was 3 or more, or as means ± S.D. (arbitrary units/μg of protein) where n was less than 3.

		PM/LDM	
Cell line	n	GLUT4	Vp165
GLUT4*	6	0.16 ± 0.03	0.03 ± 0.01
TAG^{Low}	5	0.14 ± 0.02	0.04 ± 0.02
TAG^{High}*	7	0.12 ± 0.03	0.02 ± 0.01
$TAIL^{High}$	4	2.23 ± 0.20	0.14 ± 0.09
$TELE^{Low}$	8	0.60 ± 0.12	0.15 ± 0.03
$TELE^{High}$	5	1.02 ± 0.30	0.13 ± 0.07
$YLGP^{Low}$	4	0.14 ± 0.04	0.07 ± 0.02
$YLGP^{High}$	4	1.55 ± 0.55	0.03 ± 0.01
$DEND^{Low}$	2	0.21 ± 0.03	0.02 ± 0.01
$DEND^{High}$	4	0.31 ± 0.05	0.01 ± 0.00
HA-GLUT4	5	0.28 ± 0.05	0.08 ± 0.02
HA-TAIL	3	1.09 ± 0.12	0.12 ± 0.06
HA-ELEY	4	0.88 ± 0.24	0.06 ± 0.01
HA-YLGP	4	0.66 ± 0.13	0.08 ± 0.04

* The values for endogenous GLUT4 (GLUT4) and TAG^{High} were determined previously and are presented above for the purpose of comparison [38].

In agreement with our previous studies [28,32,38], TAG was predominantly targeted to intracellular membranes in basal adipocytes, in a manner closely resembling that observed for endogenous GLUT4 and IRAP (Figure 1B and Table 1). As endogenous GLUT4 is almost entirely excluded from the cell surface and is highly enriched in the intracellular LDM fraction in non-stimulated adipocytes, the PM/LDM ratio provides a quantitative index of the degree of intracellular sequestration. We have previously reported that the PM/LDM ratio is 0.16 for GLUT4 [38], 1.3 for GLUT1 [32] and 0.12–0.33 for TAG [32,38] (Table 1). In response to insulin, the PM/LDM ratio increases to 1.2 for native GLUT4, reflecting a shift in its distribution to the cell surface (Figure 1B). Similar changes in the PM/LDM ratio of both IRAP and TAG were observed in response to insulin (Figure 1 and Table 1). In contrast with endogenous GLUT4, IRAP and TAG, a large proportion of TAIL was targeted to the cell surface in non-stimulated adipocytes (Figure 1B), as indicated by its PM/LDM ratio of 2.2 (Table 1). The cell-surface accumulation of this chimaera is not a consequence of overexpression, because TAG exhibits a normal subcellular distribution when expressed at even higher levels (Figure 1 and Table 1) [32,38]. Moreover, as shown below, the distribution of TAIL is also impaired when expressed at low levels in adipocytes. Despite this defect, TAIL translocated from the LDM to the PM fraction following insulin stimulation (Figure 1B).

Three constructs, designated TELE, YLGP and DEND, were generated by replacing amino acids in TAG, at positions 498–501 (TELE), 502–505 (YLGP) or 506–509 (DEND), with alanine residues. Detailed analysis of the subcellular distribution of the TELE mutant was performed in three clonal cell lines expressing TELE at either low or high levels of expression. TAG clones expressing recombinant GLUT4 at similar levels to TELE were

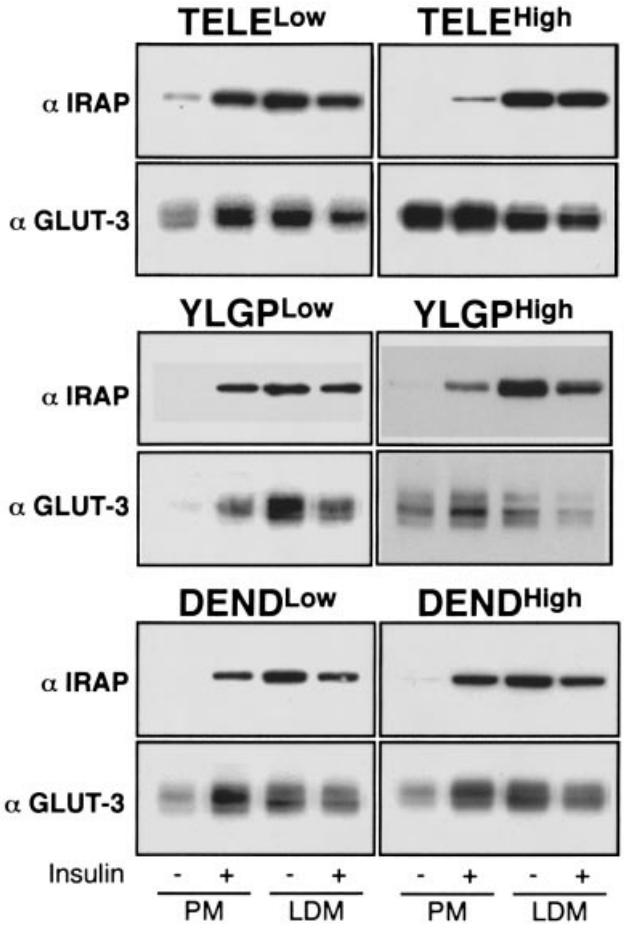

Figure 2 Subcellular distribution of C-terminal GLUT4 mutants in 3T3-L1 adipocytes

TELE, YLGP or DEND mutants were constructed in TAG and expressed in 3T3-L1 cells by stable transfection. Several different cell lines were selected, differentiated into adipocytes, incubated in the absence (−) or presence (+) of insulin and then subjected to subcellular fractionation to generate PM or LDM. These fractions were immunoblotted using antibodies specific for either the recombinant proteins ('α GLUT3') or endogenous IRAP. Shown are representative blots from cell lines expressing the various constructs at either low or high expression levels. Note that the distribution of IRAP is similar among individual cell lines. Quantification of these data is shown in Table 1.

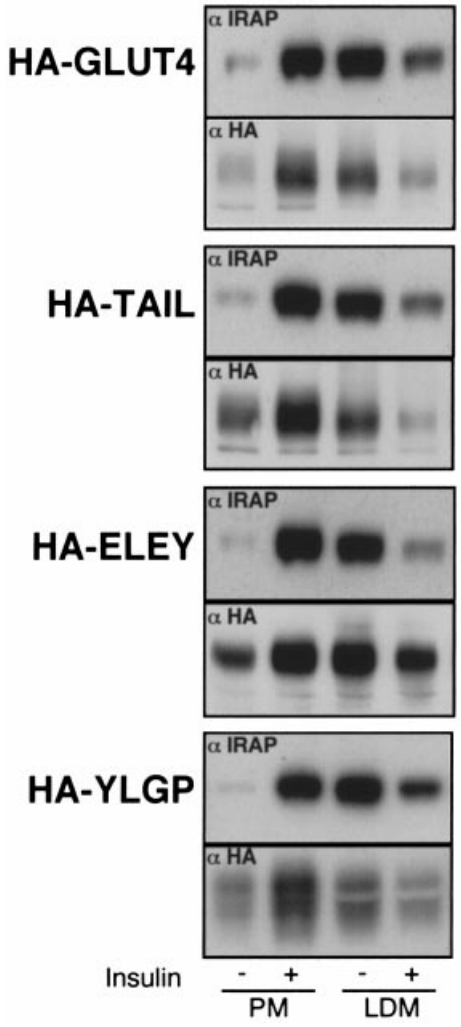

Figure 3 Subcellular-distribution analysis of exofacial HA-tagged GLUT4 (HA-GLUT4) and C-terminal GLUT4 mutants (HA-TAIL, HA-ELEY and HA-YLGP) expressed by retrovirus in 3T3-L1 adipocytes

3T3-L1 adipocyte pools expressing HA-GLUT4, HA-TAIL, HA-ELEY or HA-YLGP constructs were pre-incubated in serum-free medium for at least 2 h and further incubated for 15 min at 37 °C in the absence (−) or presence (+) of insulin. Cells were subjected to differential centrifugation, and aliquots of each fraction (10 μg of protein) immunoblotted with antibodies specific for either the HA epitope or endogenous IRAP. Data are representative for at least three separate experiments. Quantification of these data is shown in Table 1.

analysed in parallel. The distribution of TAG in clones with either low or high levels of expression was indistinguishable from endogenous GLUT4 or IRAP (Figure 1), with PM/LDM ratios in the range of 0.12–0.16. In contrast, the targeting of TELE was significantly disrupted compared with TAG, as indicated by PM/LDM ratios of $\geqslant 0.6$ (Table 1). This aberrant targeting was evident in clones expressing the mutant at both high and low levels. The subcellular distribution of IRAP in each of the TELE clones was not significantly different from that in wild-type cells (Figure 2), with PM/LDM ratios in the range of 0.10–0.18. Despite the impaired steady-state distribution of TELE in basal adipocytes, insulin still elicited the redistribution of this mutant from intracellular membranes to the cell surface (Figure 2).

The fact that the PM/LDM ratio for the TELE mutant was lower than that of TAIL (Table 1) suggested that residues distal to the TELE sequence might also contribute to the targeting information contained within the extreme C-terminus. Thus we examined the distribution of YLGP in clonal cell lines expressing the mutant at different levels. The targeting of YLGP in clones expressing the protein at levels comparable with or less than that of endogenous GLUT4 was not significantly different from TAG (Figure 2 and Table 1). However, at higher expression levels the YLGP mutant accumulated at the cell surface in basal adipocytes, as indicated by PM/LDM ratios of > 1.0 (Figure 2 and Table 1). The subcellular distribution of IRAP in cell lines expressing the YLGP mutant closely resembled that observed in other cell lines (Figures 1 and 2). Similar to TAIL and the TELE mutant, there was no demonstrable abnormality in the insulin-stimulated movement of the YLGP mutant.

Three clonal cell lines expressing the DEND mutant were also analysed (Figure 2). We were unable to find any significant change in the subcellular distribution of this mutant compared with endogenous GLUT4 or TAG, as evidenced by PM/LDM

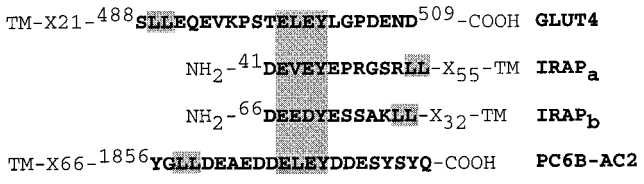

Figure 4 Alignment of consensus sequence within the cytosolic domains of GLUT4, IRAP and PC6B

The cytosolic tails of GLUT4, IRAP and PC6B contain acidic clusters membrane-distal to their respective dileucine signals. Note that IRAP is a type II membrane protein, whereas the C-termini of both GLUT4 and PC6B are cytosolic, as indicated by their topology with respect to the transmembrane ('TM') domain. IRAP contains two acid clusters in its cytosolic domain, both similarly spaced from dileucine motifs designated '$IRAP_a$' and '$IRAP_b$'. As depicted, each of the acidic clusters identified in these proteins demonstrate similar spacing (five to eight residues) between the respective dileucine motif and the acidic cluster. The area of sequence similarity within this region of each protein is shaded and constitutes a potential endosomal sorting motif, EXEY (where E is any acidic amino acid, X is any amino acid and Y is tyrosine).

ratios in the range of 0.21–0.31 (Figure 2 and Table 1). In addition, the redistribution of DEND from the intracellular fraction to the cell surface following stimulation with insulin (4.6-fold) closely resembled that observed for TAG (4.4-fold). Thus, over the range of expression levels examined here, we have been unable to discern a major contribution for the terminal four residues (DEND) in targeting GLUT4 in adipocytes.

Mutational analysis of the GLUT4 C-terminus using HA-tagged GLUT4

All of the above studies were performed using individual neomycin-resistant clones that stably expressed each construct. In order to rule out the possibility that these data were due to clonal variance rather than specific targeting defects, we constructed a similar set of mutants in the context of a HA-tagged GLUT4 construct [33]. In addition, all constructs were introduced into 3T3-L1 cells by retroviral infection to generate polyclonal pools of 3T3-L1 adipocytes expressing each construct [34]. The differentiation efficiency of 3T3-L1 cells was unaffected by retroviral infection, and the level of expression achieved for glucose-transporter constructs was generally lower than endogenous levels. In addition, we found that the expression of all constructs was quite uniform in adipocytes using this system (results not shown). Thus it is highly unlikely that any targeting defect observed for HA-tagged GLUT4 constructs is due to overexpression *per se*. Furthermore, by using a construct in which the HA epitope had been engineered into the large exofacial loop of wild-type GLUT4 [33], we circumvented potential problems associated with introduction of the GLUT3 epitope tag at the end of full-length GLUT4 as employed in our original strategy. The basal distribution of HA-tagged GLUT4 (HA-GLUT4) resembled both endogenous GLUT4 and TAG, with the protein largely excluded from the plasma membrane and enriched in the intracellular LDM fraction (Figure 3). In contrast, HA-TAIL showed significant accumulation at the cell surface in the basal state (Figure 3), as indicated by a PM/LDM ratio of 1.1. Despite this difference, HA-TAIL retained the ability to translocate to the plasma membrane in response to insulin. These data are consistent with the studies described above using clonal cell lines (Figure 1).

Using the same approach, we analysed the subcellular distribution of two GLUT4 mutants in which the residues $ELEY^{499–502}$ or $YLGP^{502–505}$ were mutated *en bloc* to alanine residues. In these studies we opted to target the residues ELEY because of sequence similarity with the cytosolic tail of IRAP (see Figure 4). In agreement with the studies in stable cell lines we observed a significant increase in the steady-state accumulation of both HA-ELEY and HA-YLGP at the cell surface in the absence of insulin (Figure 3). This was indicated by PM/LDM ratios of 0.88 and 0.66 for HA-ELEY and HA-YLGP respectively (Table 1). In contrast, the subcellular distribution of IRAP in polyclonal pools expressing each of these constructs was identical with that found in control cells with PM/LDM ratios of 0.09–0.14 (Figure 3 and Table 1).

Table 2 Endosomal ablation analysis of C-terminal GLUT4 mutants

3T3-L1 adipocytes were incubated with Tf–HRP conjugate for 3 h at 37 °C followed by the addition of 3,3′-diaminobenzene, in the absence or presence of H_2O_2. Intracellular membranes were prepared and immunoblotted with antibodies specific for either the GLUT3 epitope or the C-terminus of GLUT4. The extent of protein ablation was determined from the difference in immunoreactive signals between cells incubated ± peroxide. Shown below is the signal remaining in the LDM after ablation expressed as a percentage of the signal in the LDM before ablation. As a control in all these experiments, cells were incubated with Tf–HRP, 3,3′-diaminobenzidine and H_2O_2 at 4 °C in parallel. Under these conditions, no internalization of Tf–HRP is expected, and, consistent with this, we observed no ablation of either endogenous or recombinant GLUT4 (results not shown). Quantitative data are presented for individual cell lines classified as either low or high based on the level of expression of the corresponding GLUT4 construct. Values are expressed as means ± S.E.M. (arbitrary units/μg of protein) where n is 3 or more or as means ± S.D. (arbitrary units/μg of protein) where n is less than 3. n represents the number of independent experiments.

Cell line	n	Non-ablated (%)
GLUT4*	3	59 ± 5
TAG^{Low}	3	56 ± 1
TAG^{High}*	3	60 ± 5
$TELE^{Low}$	2	17 ± 3
$TELE^{Low}$	2	15 ± 1
$TELE^{High}$	2	16 ± 1
$YLGP^{Low}$	4	60 ± 0
$YLGP^{High}$	2	59 ± 1
$YLGP^{High}$	2	50 ± 5
$DEND^{High}$	2	54 ± 4
$DEND^{High}$	3	56 ± 1

* The values for endogenous GLUT4 (GLUT4) and TAG^{High} were determined previously.

Endosomal-ablation analysis

It has been previously shown that GLUT4 is distributed between multiple intracellular compartments in insulin-sensitive cells [40]. Using a technique in which Tf–HRP is used to selectively 'ablate' the Tf-receptor-positive recycling compartment of 3T3-L1 adipocytes, it is possible to segregate intracellular GLUT4 membranes into two distinct entities [37]. One pool, presumably consisting of recycling endosomes, is accessible to the recycling Tf–HRP conjugate, and comprises $\approx 40\%$ of the total GLUT4 in this fraction. The non-ablated portion contains the remaining 60% of intracellular GLUT4, and most likely represents the highly insulin-responsive pool [37]. As early endosomal markers are not enriched in this fraction, we have referred to this pool as a 'post-endocytic GLUT4 storage compartment' [5]. To determine if the sequence under investigation functions in the intracellular sorting of GLUT4, we analysed the C-terminal mutants generated in the present study by Tf–HRP ablation. The sensitivity of epitope-tagged GLUT4 (TAG) to chemical ablation following a 3 h incubation with the Tf–HRP conjugate at 37 °C

(44 %) (Table 2) was indistinguishable from wild-type GLUT4 (41 %) [28,37,38]. This suggests that TAG is partitioned normally between endosomes and a post-endocytic compartment. Similar results were obtained with cell lines expressing either the YLGP or DEND mutants. In contrast, the TELE mutant was significantly ablated following either a 1 h (58 %) (results not shown) or 3 h (84 %) incubation with the Tf–HRP conjugate at 37 °C (Table 2). Consistent with our previous findings [28,37,38], membranes blotted in parallel with antibodies specific for the Tf receptor, cellubrevin or GLUT1 showed > 85 % ablation of these proteins after incubation for 3 h at 37 °C with the Tf–HRP conjugate, for all of the cell lines studied (results not shown).

DISCUSSION

In the absence of insulin GLUT4 is sequestered within an intracellular storage compartment in both muscle and adipose cells. Insulin overcomes this sequestration resulting in increased cell-surface levels of GLUT4 [3,40]. Kinetic analysis of GLUT4 recycling in adipocytes reveals that it has a very slow exocytosis rate in the absence of insulin compared with other recycling proteins, such as the transferrin receptor [4,41]. This suggests that within the cell GLUT4 is sorted away from the bulk of endosomal traffic, thus preventing its entry into constitutively recycling transport vesicles. Dissecting the molecular basis of this intracellular sequestration promises to offer new insight into insulin action. Trafficking within the endosomal system appears to be regulated by sorting signals in the cytosolic tails of recycling membrane proteins [8,9]. These signals are recognized by sorting machinery which concentrate and sort these molecules into specific transport vesicles [10]. A number of laboratories have attempted to identify sorting signals in cytosolic domains of GLUT4 by constructing either chimaeric proteins or point mutants. This has led to the identification of two separate motifs: FQQI in the N-terminus and a dileucine motif in the C-terminal tail [21–23]. Both of these motifs function autonomously to regulate internalization from the cell surface, and similar types of motifs have been found to regulate internalization in other recycling proteins [22,25–27]. While it remains unclear how these signals might regulate other aspects of GLUT4 trafficking [28], there is reason to believe that alternate trafficking signals exist in GLUT4. Analysis of GLUT1/GLUT4 chimaeras in either L6 myoblasts or 3T3-L1 adipocytes clearly indicates an important role for the GLUT4 C-terminus in targeting the protein to a highly insulin-responsive compartment [27,29]. However, mutation of the dileucine signal in this tail did not disrupt this targeting function [29].

In the present study we have carried out a functional analysis of the extreme C-terminus of GLUT4 in 3T3-L1 adipocytes. We provide evidence for an additional targeting signal, comprised of the residues TELEYLGP, distal to the dileucine motif. Mutation of various residues within this motif results in constitutive accumulation of GLUT4 at the PM. Using a technique to ablate endosomes in 3T3-L1 adipocytes, we also show that mutants in which this motif has been disrupted accumulate in endosomes, suggesting that this signal may regulate a discrete targeting step within the endosomal recycling system.

It has been suggested by both ourselves and others that GLUT4 may be sorted out of recycling endosomes into a post-endocytic GLUT4 pool that is highly insulin responsive [5,37,40]. We propose that the TELEYLGP sequence may regulate this step. It is not clear from our studies if the TELEYLGP signal operates independently of the dileucine signal. Indeed, it is feasible that there are interactions between the N- and C-termini of GLUT4, thus potentially implicating some type of interaction between this motif and the FQQI signal. It is notable that similar types of targeting signals, referred to as 'acidic clusters', have been identified in a variety of other membrane proteins. These include the low-density-lipoprotein receptor [9], IRAP [39], furin [42,43], the proprotein convertase PC6B [44], and the cation-dependent and cation-independent MPRs [11,13]. These motifs appear to be generally involved in regulating trafficking between the TGN and endosomes. Intriguingly, these motifs are often flanked by either tyrosine-based or dileucine targeting signals, and in some cases it has been shown that there is some interdependence between these signals with respect to targeting [44]. As shown in Figure 4, the acidic cluster motif in the C-terminus of GLUT4 is distal, with respect to the transmembrane domain, from the dileucine signal. Most notably the cytosolic tail of IRAP contains two dileucine signals, both of which are separated from a putative acidic cluster motif by a similar spacing to that observed in GLUT4 (Figure 4). In addition, in both cases the topology of these signals with respect to the lipid bilayer is preserved. In a recent study [44] examining the targeting of a member of the proprotein convertase family, known as PC6B, two acidic clusters have been identified. The first of these (AC1) regulates localization to the TGN; this motif interacts with the TGN sorting protein PACS-1. The second motif (AC2) regulates traffic of PC6B to a regulatable secretory-type compartment that is connected to endosomes. This trafficking pattern has a number of similarities with that previously observed for GLUT4 [5,37]. Strikingly, there is considerable sequence similarity between the acidic cluster that we have defined in the present study and AC2 in the tail of PC6B (Figure 4), including the spacing with respect to a dileucine signal. In contrast with AC1, AC2 does not interact with PACS-1 [44]. This is of interest because the GLUT4 C-terminus also does not interact with PACS-1 (S. Rea and D. E. James, unpublished work). Hence, it will be of considerable interest to identify the binding partner for the sorting motif identified in the present study and to determine if it also interacts with PC6B and possibly the tail of IRAP.

In the future it will be important to define the precise amino acids that comprise the C-terminal GLUT4 targeting motif. The acidic residues at positions 499 and 501 do not appear to comprise the entire motif, because we also observed impaired targeting of the YLGP mutant (Figures 2 and 3). Hence, on the basis of these data and the sequence similarity shown in Figure 4, it seems evident that there is some role for the tyrosine at position 502. We have recently examined the structure of the GLUT4 C-terminus using two-dimensional NMR. These studies indicate that while most of the tail is relatively unstructured, there is significant nascent helix around the dileucine and residues 498–500 with the potential for turn elements immediately adjacent to the acidic cluster (A. Atkins, J. Wade and D. Craik, personal communication). Although these studies did not provide evidence of structural interaction between the acidic cluster and the dileucine signal, it is possible, as for the low-density-lipoprotein receptor, that this may comprise an overlapping motif, different elements of which may regulate discrete targeting events [17]. It has been shown that a synthetic peptide comprising the dileucine signal in the C-terminus of GLUT4, but not the intact TELEYLGP sequence, interacts with the AP1 adaptor complex [45]. In addition, we have shown that GLUT4 co-localizes with AP1 in adipocytes, and this interaction can be modified by mutating the adjacent serine at position 488 [38]. Thus it seems possible that the GLUT4 dileucine signal may participate in three separate targeting steps: internalization, TGN sorting via AP1 and endosomal sorting. It would appear, however, that if the acidic-cluster domain operates in tandem

with the dileucine, it must be dominant, because the phenotype we observe in mutants in which the acidic cluster has been mutated are much more severe than those bearing dileucine mutations [27,32].

The most significant challenge for the future is to define the function of these different targeting motifs. It has previously been proposed that GLUT4 enters the endosomal system together with the transferrin receptor and is then sorted into a post-endocytic storage compartment from where it may move to the cell surface with insulin [46]. On the basis of our endosomal-ablation data we propose that the C-terminal acidic cluster motif may play an important role in this step. The identification of similar types of motif in the tail of IRAP and PC6B support this concept. On the basis of the sequence similarity shown in Figure 4, it will be intriguing to determine if PC6B and GLUT4 cycle through the same intracellular compartment(s) in basal adipocytes. Defining the biogenesis of this post-endocytic storage compartment, and how it relates to the endo-lysosomal system should provide insight into the multiple targeting mechanisms at work within GLUT4 cytosolic domains.

We thank Teresa Munchow for tissue-culture expertise. This work was supported by the National Health and Medical Research Council (NHMRC) of Australia, The British Diabetic Association and the Medical Research Council (U.K.). The Institute for Molecular Bioscience is a Special Research Centre of the Australian Research Council.

REFERENCES

1 Simonsen, A., Stang, E., Bremnes, B., Roe, M., Prydz, K. and Bakke, O. (1997) Sorting of MHC class II molecules and the associated invariant chain (Ii) in polarized MDCK cells. J. Cell Sci. **110**, 597–609
2 Slot, J. W., Geuze, H. J., Gigengack, S., Lienhard, G. E. and James, D. E. (1991) Immuno-localization of the insulin regulatable glucose transporter in brown adipose tissue of the rat. J. Cell Biol. **113**, 123–135
3 Slot, J. W., Geuze, H. J., Gigengack, S., James, D. E. and Lienhard, G. E. (1991) Translocation of the glucose transporter GLUT4 in cardiac myocytes of the rat. Proc. Natl. Acad. Sci. U.S.A. **88**, 7815–7819
4 Yang, J. and Holman, G. D. (1993) Comparison of GLUT4 and GLUT1 subcellular trafficking in basal and insulin-stimulated 3T3–L1 cells. J. Biol. Chem. **268**, 4600–4603
5 Martin, S., Tellam, J., Livingstone, C., Slot, J. W., Gould, G. W. and James, D. E. (1996) The glucose transporter (GLUT-4) and vesicle-associated membrane protein-2 (VAMP-2) are segregated from recycling endosomes in insulin-sensitive cells. J. Cell Biol. **134**, 625–635
6 Bremnes, T., Lauvrak, V., Lindqvist, B. and Bakke, O. (1998) A region from the medium chain adaptor subunit (mu) recognizes leucine- and tyrosine-based sorting signals. J. Biol. Chem. **273**, 8638–8645
7 Rodionov, D. G. and Bakke, O. (1998) Medium chains of adaptor complexes AP-1 and AP-2 recognize leucine-based sorting signals from the invariant chain. J. Biol. Chem. **273**, 6005–6008
8 Marks, M. S., Woodruff, L., Ohno, H. and Bonifacino, J. S. (1996) Protein targeting by tyrosine- and di-leucine-based signals: evidence for distinct saturable components. J. Cell Biol. **135**, 341–354
9 Mellman, I. (1996) Endocytosis and molecular sorting. Annu. Rev. Cell Dev. Biol. **12**, 575–625
10 Hirst, J. and Robinson, M. S. (1998) Clathrin and adaptors. Biochim. Biophys. Acta **1404**, 173–193
11 Johnson, K. F. and Kornfeld, S. (1992) The cytoplasmic tail of the mannose 6-phosphate/insulin-like growth factor-II receptor has two signals for lysosomal enzyme sorting in the Golgi. J. Cell Biol. **119**, 249–257
12 Le Borgne, R., Schmidt, A., Mauxion, F., Griffiths, G. and Hoflack, B. (1993) Binding of AP-1 Golgi adaptors to membranes requires phosphorylated cytoplasmic domains of the mannose 6-phosphate/insulin-like growth factor II receptor. J. Biol. Chem. **268**, 22552–22556
13 Glickman, J. N., Conibear, E. and Pearse, B. M. (1989) Specificity of binding of clathrin adaptors to signals on the mannose-6- phosphate/insulin-like growth factor II receptor. EMBO J. **8**, 1041–1047
14 Zhang, Y. and Allison, J. P. (1997) Interaction of CTLA-4 with AP50, a clathrin-coated pit adaptor protein. Proc. Natl Acad. Sci. U.S.A. **94**, 9273–9278
15 Honing, S., Sandoval, I. V. and von Figura, K. (1998) A di-leucine-based motif in the cytoplasmic tail of LIMP-II and tyrosinase mediates selective binding of AP-3. EMBO J. **17**, 1304–1314
16 Geisler, C., Dietrich, J., Nielsen, B. L., Kastrup, J., Lauritsen, J. P., Odum, N. and Christensen, M. D. (1998) Leucine-based receptor sorting motifs are dependent on the spacing relative to the plasma membrane. J. Biol. Chem. **273**, 21316–21323
17 Matter, K., Yamamoto, E. M. and Mellman, I. (1994) Structural requirements and sequence motifs for polarized sorting and endocytosis of LDL and Fc receptors in MDCK cells. J. Cell Biol. **126**, 991–1004
18 Marshall, B. A., Murata, H., Hresko, R. C. and Mueckler, M. (1993) Domains that confer intracellular sequestration of the Glut4 glucose transporter in *Xenopus* oocytes. J. Biol. Chem. **268**, 26193–26199
19 Piper, R. C., Tai, C., Slot, J. W., Hahn, C. S., Rice, C., Huang, H. and James, D. E. (1992) The efficient intracellular sequestration of the insulin-regulatable glucose transporter (GLUT4) is conferred by the N terminus. J. Cell Biol. **117**, 729–743
20 Verhey, K. J., Hausdorff, S. F. and Birnbaum, M. J. (1993) Identification of the carboxy-terminus as important for the isoform-specific subcellular targeting of glucose transporter proteins. J. Cell Biol. **123**, 137–147
21 Corvera, S., Chawla, A., Chakrabarti, R., Joly, M., Buxton, J. and Czech, M. P. (1994) A double leucine within the GLUT4 glucose transporter COOH-terminal domain functions as an endocytosis signal. J. Cell Biol. **126**, 979–989
22 Piper, R. C., Tai, C., Kulesza, P., Pang, S., Warnock, D., Baenziger, J., Slot, J. W., Geuze, H. J., Puri, C. and James, D. E. (1993) GLUT-4 NH2 terminus contains a phenylalanine-based targeting motif that regulates intracellular sequestration. J. Cell Biol. **121**, 1221–1232
23 Verhey, K. J. and Birnbaum, M. J. (1994) A Leu-Leu sequence is essential for COOH-terminal targeting signal of GLUT4 glucose transporter in fibroblasts. J. Biol. Chem. **269**, 2353–2356
24 Kao, A. W., Ceresa, B. P., Santeler, S. R. and Pessin, J. E. (1998) Expression of a dominant interfering dynamin mutant in 3T3L1 adipocytes inhibits GLUT4 endocytosis without affecting insulin signaling. J. Biol. Chem. **273**, 25450–25457
25 Garippa, R. J., Judge, T. W., James, D. E. and McGraw, T. E. (1994) The amino terminus of GLUT-4 functions as an internalisation motif but not an intracellular retention signal when substituted for the transferrin receptor cytoplasmic domain. J. Cell Biol. **124**, 705–716
26 Garippa, R. J., Johnson, A., Park, J., Petrush, R. L. and Mcgraw, T. E. (1996) The carboxyl terminus of GLUT4 contains a Serine-Leucine-Leucine sequence that functions as a potent internalization motif in chinese hamster ovary cells. J. Biol. Chem. **271**, 20660–20668
27 Verhey, K. J., Yeh, J. I. and Birnbaum, M. J. (1995) Distinct signals in the GLUT4 glucose transporter for internalization and for targeting to an insulin-responsive compartment. J. Cell Biol. **130**, 1071–1079
28 Melvin, D. R., Marsh, B. J., Walmsley, A. R., James, D. E. and Gould, G. W. (1999) Analysis of amino and carboxy terminal GLUT-4 targeting motifs in 3T3–L1 adipocytes using an endosomal ablation technique. Biochemistry **38**, 1456–1462
29 Haney, P. M., Levy, M. A., Strube, M. S. and Mueckler, M. (1995) Insulin-sensitive targeting of the GLUT4 glucose transporter in L6 myoblasts is conferred by its COOH-terminal cytoplasmic tail. J. Cell Biol. **129**, 641–658
30 James, D. E., Strube, M. and Mueckler, M. (1989) Molecular cloning and characterization of an insulin regulatable glucose transporter. Nature (London) **338**, 83–87
31 Harris, D. S., Slot, J. W., Geuze, H. J. and James, D. E. (1992) Polarized distribution of glucose transporter isoforms in Caco-2 cells. Proc. Natl. Acad. Sci. U.S.A. **89**, 7556–7560
32 Marsh, B. J., Alm, R. A., McIntosh, S. R. and James, D. E. (1995) Molecular regulation of GLUT-4 targeting in 3T3–L1 adipocytes. J. Cell Biol. **130**, 1081–1091
33 Quon, M. J., Guerre-Millo, M., Zarnowski, M. J., Butte, A. J., Em, M., Cushman, S. W. and Taylor, S. I. (1994) Tyrosine kinase-deficient mutant human insulin receptors ($Met^{1153} \rightarrow Ile$) overexpressed in transfected rat adipose cells fail to mediate translocation of epitope-tagged GLUT4. Proc. Natl. Acad. Sci. U.S.A. **91**, 5587–5591
34 Morgenstern, J. P. and Land, H. (1990) Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res. **18**, 3587–3596
35 Pear, W. S., Nolan, G. P., Scott, M. L. and Baltimore, D. (1993) Production of high-titer helper-free retroviruses by transient transfection. Proc. Natl Acad. Sci. U.S.A. **90**, 8392–8396
36 Piper, R. C., Hess, L. J. and James, D. E. (1991) Differential sorting of two glucose transporters expressed in insulin-sensitive cells. Am. J. Physiol. **260**, C570–C580
37 Livingstone, C., James, D. E., Rice, J. E., Hanpeter, D. and Gould, G. W. (1996) Compartment ablation analysis of the insulin-responsive glucose transporter (GLUT4) in 3T3–L1 adipocytes. Biochem. J. **315**, 487–495

38 Marsh, B. J., Martin, S., Melvin, D. R., Martin, L. B., Alm, R. A., Gould, G. W. and James, D. E. (1998) Mutational analysis of the carboxy-terminal phosphorylation site of GLUT-4 in 3T3–L1 adipocytes. Am. J. Physiol. **275**, E412–E422
39 Ross, S. A., Scott, H. M., Morris, N. J., Leung, W. Y., Mao, F., Lienhard, G. E. and Keller, S. R. (1996) Characterization of the insulin-regulated membrane aminopeptidase in 3T3–L1 adipocytes. J. Biol. Chem. **271**, 3328–3332
40 Slot, J. W., Moxley, R., Geuze, H. J. and James, D. E. (1990) Distribution of the insulin-regulatable glucose transporters in muscle and adipose tissue. Nature (London) **346**, 369–371
41 Tanner, L. I. and Lienhard, G. E. (1987) Insulin elicits a redistribution of transferrin receptors in 3T3–L1 adipocytes through an increase in the rate constant for receptor externalization. J. Biol. Chem. **262**, 8975–8980
42 Schafer, W., Stroh, A., Berghofer, S., Seiler, J., Vey, M., Kruse, M. L., Kern, H. F., Klenk, H. D. and Garten, W. (1995) Two independent targeting signals in the cytoplasmic domain determine *trans*-Golgi network localization and endosomal trafficking of the proprotein convertase furin. EMBO J. **14**, 2424–2435
43 Voorhees, P., Deignan, E., van Donselaar, E., Humphrey, J., Marks, M. S., Peters, P. J. and Bonifacino, J. S. (1995) An acidic sequence within the cytoplasmic domain of furin functions as a determinant of *trans*-Golgi network localization and internalization from the cell surface. EMBO J. **14**, 4961–4975
44 Xiang, Y., Molloy, S. S., Thomas, L. and Thomas, G. (2000) The PC6B cytoplasmic domain contains two acidic clusters that direct sorting to distinct *trans*-Golgi network/endosomal compartments. Mol. Biol. Cell. **11**, 1257–1273
45 Rapoport, I., Chen, Y. C., Cupers, P., Shoelson, S. E. and Kirchhausen, T. (1998) Dileucine-based sorting signals bind to the beta chain of AP-1 at a site distinct and regulated differently from the tyrosine-based motif-binding site. EMBO J. **17**, 2148–2155
46 Martin, L. B., Shewan, A. M., Millar, C. A., Gould, G. W. and James, D. E. (1998) Vesicle-associated membrane protein 2 plays a specific role in the insulin-dependent trafficking of the facilitative glucose transporter GLUT4 in 3T3-L1 adipocytes. J. Biol. Chem. **273**, 1444–1452

Received 4 January 2000/25 April 2000; accepted 29 May 2000