An essential role for the Glut1 PDZ-binding motif in growth factor regulation of Glut1 degradation and trafficking

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Cell surface localization of the Glut (glucose transporter), Glut1, is a cytokine-controlled process essential to support the metabolism and survival of haemopoietic cells. Molecular mechanisms that regulate Glut1 trafficking, however, are not certain. In the present study, we show that a C-terminal PDZ-binding motif in Glut1 is critical to promote maximal cytokine-stimulated Glut1 cell surface localization and prevent Glut1 lysosomal degradation in the absence of growth factor. Disruption of this PDZ-binding sequence through deletion or point mutation sharply decreased surface Glut1 levels and led to rapid targeting of internalized Glut1 to lysosomes for proteolysis, particularly in growth factor-deprived cells. The PDZ-domain protein, GIPC (Gα-interacting protein-interacting protein, C-terminus), bound to Glut1 in part via the Glut1 C-terminal PDZ-binding motif, and we found that GIPC deficiency decreased Glut1 surface levels and glucose uptake. Unlike the Glut1 degradation observed on mutation of the Glut1 PDZ-binding domain, however, GIPC deficiency resulted in accumulation of intracellular Glut1 in a pool distinct from the recycling pathway of the TfR (transferrin receptor). Blockade of Glut1 lysosomal targeting after growth factor withdrawal also led to intracellular accumulation of Glut1, a portion of which could be rapidly restored to the cell surface after growth factor stimulation. These results indicate that the C-terminal PDZ-binding motif of Glut1 plays a key role in growth factor regulation of glucose uptake by both allowing GIPC to promote Glut1 trafficking to the cell surface and protecting intracellular Glut1 from lysosomal degradation after growth factor withdrawal, thus allowing the potential for a rapid return of intracellular Glut1 to the cell surface on restimulation.

Key words: Gα-interacting protein-interacting protein (GIPC), glucose transporter (Glut), growth factor, lysosomal degradation, PDZ-binding motif, small-hairpin RNA interference (shRNAi).

INTRODUCTION

Cellular metabolic rates must meet biosynthetic and energetic demands to allow cell survival and function. Thus rapidly growing and proliferating cells, such as growth factor-stimulated or cancer cells, require increased metabolism relative to their non-proliferating counterparts. Glucose, in particular, is preferentially utilized by proliferating cells for biosynthesis and energy production. Resting haemopoietic cells normally utilize glucose at a low rate until stimulated by a specific growth or mitogenic factor, such as the cytokines IL-3 (interleukin-3) or IL-7, which can promote dramatic increases in glucose uptake and consumption [1–3]. Cells deprived of these growth factors undergo a sharp decrease in glucose metabolism, initiating cellular atrophy and eventually cell death [1,4–7]. Glucose uptake is a key regulating factor of cell growth and survival as glucose metabolism can both provide energy for cell growth and influence Bcl-2 family protein regulation of cell death [6,13,14]. Rapid loss of Glut1 protein after growth factor deprivation may prevent cells from responding quickly to re-stimulation, whereas inappropriate recycling of intracellular Glut1 may allow continued glucose uptake to prevent apoptosis after growth factor withdrawal [6].

It has been suggested that the C-terminal tail of Glut1 is critical to control Glut1 subcellular localization [15] and may mediate protein interactions essential for cytokine-regulated Glut1 trafficking. Yeast two-hybrid screens have identified two
proteins that bind to the Glut1 C-terminal tail which may control Glut1 trafficking, namely a SUMO (small ubiquitin-related modifier)-conjugating enzyme, Ubc9, and the PDZ-domain protein, GIPC (G-β/δ-interacting protein-interacting protein, C-terminus) [16,17]. Ubc9 has been shown to interact with a specific lysine residue on the C-terminal tail of Glut1 and Glut4, but not Glut3, and to influence total Glut1 and Glut4 protein levels [17]. In addition, the C-terminal four amino acids of Glut1 (DSQV) represent a class I PDZ-binding motif [18,19] that is required for efficient GIPC–Glut1 interaction [16]. GIPC also associates with and promotes cell surface localization of other cell surface proteins, including transforming growth factor-type III receptor, human lutropin receptors and dopamine D2 and D3 receptors [20–22]. Co-localization studies of Glut1 lacking the PDZ-binding motif have suggested that GIPC may protect Glut1 from association with late endosomes/lysosomes [23].

To better understand cytokine regulation of glucose uptake and Glut1 lysosomal targeting, we have analysed the role of specific domains of Glut1 required for growth factor-regulated Glut1 trafficking and glucose uptake. In the present study, we show that the Glut1 C-terminal PDZ-binding motif plays critical roles in regulation of Glut1 trafficking. Importantly, internalized Glut1 was rapidly targeted for lysosomal degradation on growth factor withdrawal when the Glut1 PDZ-binding motif was deleted or mutated. The Glut1-binding PDZ-domain protein GIPC, however, did not regulate Glut1 lysosomal targeting. Instead, GIPC was required to promote return of intracellular Glut1 to the cell surface as total Glut1 levels were unaltered in GIPC-deficient cells, resulting in Glut1 accumulation in an intracellular pool distinct from the endosomal recycling pathway characterized by TIR (transferrin receptor) localization. Prevention of Glut1 targeting to lysosomes also maintained Glut1 protein levels on growth factor withdrawal with accumulation of intracellular Glut1. Importantly, a portion of this intracellular Glut1 could be rapidly restored to the cell surface on restimulation. Taken together, these results show that the PDZ-binding motif of Glut1 plays a key role in regulating Glut1 protein levels and trafficking to both the cell surface and the lysosomes and in supporting growth factor-regulated glucose uptake and cell growth.

MATERIALS AND METHODS

Plasmids

N-terminal GFP (green fluorescent protein)-tagged hGlut3 (human Glut3) was generated as previously described for GFP–rGlut1 (rat Glut1) [10]. Mutants of FLAG–rGlut1 were generated to delete the last 38 amino acids or the last four amino acids using the FLAG-tagged rGlut1 described previously [10]. A FLAG-tagged chimaeric protein was constructed by replacing the rGlut1 C-terminal tail (38 amino acids) with the corresponding C-terminal tail of hGlut3 (44 amino acids). Point mutagenesis was used to mutate Lys-456 of FLAG–Glut1 to an alanine residue. A double mutant was constructed by deleting the C-terminal four amino acids of the FLAG–Glut1K456A construct. Point mutagenesis was used to mutate individual amino acids in the PDZ-binding domain of Glut1 (DSQV) to alanine residues as indicated. mRab7 (mouse Rab7) shRNAi (small-hairpin RNAi) plasmid was constructed using pCR2.1 TOPO (Invitrogen, Carlsbad, CA, U.S.A.) with the hU6 promoter and published target sequence [11]. pET30a (+)-GIPC vector for expression of recombinant His₆-tagged GIPC was generously provided by Dr Brent Reed (Louisiana State University, Shreveport, LA, U.S.A.). mGIPC (mouse GIPC) shRNAi plasmids were constructed using pCR2.1 TOPO cloning (Invitrogen) with the hU6 promoter and target sequences to 486–504 bp or 570–593 bp of mGIPC. GFP shRNAi was utilized as a control plasmid for down-regulation [6]. A mGIPC overexpression construct was made by reverse transcription of mouse T-cell cDNA and cloned into pCR2.1 TOPO before subcloning into pEF6 vector. pEF6-myrAkt1 has been previously described [10].

Cells

The early haemopoietic myeloid/lymphoid cell line, FL5.12, was cultured as previously described [10,24] with addition of recombinant murine IL-3 (500 pg/ml; Peprotech, Rocky Hill, NJ, U.S.A.). For inhibition of lysosomal degradation, cells were cultured in either 40 μM chloroquine (Sigma–Aldrich, St Louis, MO, U.S.A.) or 10 mM ammonium chloride (NH₄Cl) (Sigma–Aldrich) for the duration of growth factor withdrawal. Stable expression of FLAG–Glut1, FLAG–Glut1ΔC, FLAG–Glut1Δ4, FLAG–Glut1CtermGlut3, FLAG–Glut1K456A and FLAG–Glut1K456AΔ4 in FL5.12 cells was achieved by transfection of a linearized construct by electroporation (Kit V; Amaxa Biosystems, Gaithersburg, MD, U.S.A.). Stable clones were identified after selection with blasticidin (Invitrogen) and flow cytometric analysis for surface FLAG-positive cells. Stable expression of Bcl-XL in FLAG–Glut1 cells was achieved by retroviral transfection of pKDs-GFP-hBcl-xL. Bulk populations of cells were used, gating on GFP-positive cells. For growth factor withdrawal, cells were washed three times in PBS prior to resuspension in appropriate medium for 6 h unless otherwise noted.

2-Deoxy-o-glucose transport assay

Glucose uptake was measured as previously described [10]. Briefly, cells were resuspended in KRH (Kreb’s Ringer Hepes) (KRH at pH 7.4: 136 mM NaCl, 4.7 mM KCl, 1.25 mM CaCl₂, 1.25 mM MgSO₄, and 10 mM HEPES). 2-Deoxy-D-[1-¹⁴C]glucose (2 μ Ci/reaction) was added for a period of 5 min at 37°C. Reactions were quenched by the addition of ice-cold 200 μM phloretin (Calbiochem, San Diego, CA, U.S.A.) and centrifugation through an oil layer [1:1 Dow Corning 550 silicon fluid (Motion Industries, Birmingham, AL, U.S.A.) and dioctyl phthalate (Sigma–Aldrich)]. The oil layer was washed and the cell pellet was solubilized in 1 M NaOH and radioactivity was measured with a scintillation counter.

Flow cytometry

Cells were analysed by a FACSscan (Becton Dickinson, San Jose, CA, U.S.A.) and FlowJo software (Tree Star, Ashland, OR, U.S.A.). To determine FLAG surface expression, cells were stained and analysed as previously described [10]. To measure internalization of FLAG–Glut1, pulse–chase assays were performed as described previously [10]. To determine TIR (CD71) surface expression, cells were incubated with anti-mouse CD71-PE (BD Biosciences PharMingen, San Diego, CA, U.S.A.) for 20 min on ice.

Fluorescence microscopy

To image cells expressing GFP fusion proteins, cells were fixed with 1% (w/v) paraformaldehyde in PBS and viewed with a Zeiss LSM410 confocal microscope (Carl Zeiss, Thornwood, NY, U.S.A.) and MetaMorph software (Molecular Devices, Sunnyvale, CA, U.S.A.). To image lysosomes, cells were treated with 50 nM Lysotracker Red (Invitrogen) for 30 min before fixation. To image TIR trafficking, cells were treated with 50 μg/ml Alexa Fluor® 568-tagged transferrin for 1 h before fixation (Invitrogen).
Preparation of His₆–GIPC and pulldowns

The pET30a(+)–GIPC vector was transformed into Escherichia coli BL21DE3 pLys cells and His₆–GIPC was purified as described in [16]. Briefly, cells were induced and fusion protein was purified from inclusion bodies with Ni-affinity chromatography (Ni-NTA (Ni²⁺-nitrilotriacetate)-agarose; Qiagen, Valencia, CA, U.S.A.) under denaturing conditions using guanidinium chloride. The nickel-bound His₆–GIPC beads were washed extensively with a buffer containing 10 mM Hepes (pH 8.0), 1 M NaCl and 1 mM DTT (dithiothreitol). Control nickel beads were preblocked in 0.125 M histidine followed by additional blocking for both control and His₆–GIPC beads in PBS/3% (w/v) BSA. Beads were then washed with PBS/1% (v/v) Triton X-100 before overnight incubation with lysates from cells transfected to transiently express FLAG–Glut1 proteins. Beads were resuspended in SDS sample buffer and heated at 70°C for 10 min before loading on to SDS/PAGE gels.

Western blot analysis

For Western blotting cells were lysed for 1 h on ice in 1% (v/v) Triton X-100 and 0.1% SDS in PBS in presence of protease inhibitors (BD Biosciences PharMingen) and precleared by centrifugation. Primary antibodies used were: anti-FLAG M2 peroxidase (Sigma–Aldrich); rabbit anti-FLAG 1 (Abcam, Cambridge, MA, U.S.A.); rabbit anti-phospho-Akt Ser-473 (Cell Signaling Technology, Beverly, MA, U.S.A.); mouse anti-actin (Sigma); goat anti-GIPC (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.); rabbit anti-6×His (Abcam); and mouse anti-Rab7 (Santa Cruz Biotechnology). Secondary antibodies used were: anti-rabbit HRP (horseradish peroxidase) (Cell Signaling Technology); anti-mouse HRP (BD Biosciences PharMingen); and donkey anti-goat HRP (Santa Cruz Biotechnology). Secondary HRP-conjugated antibodies were viewed by ECL®–Plus (GE Healthcare, Little Chalfont, Buckinghamshire, U.K.).

RESULTS

The Glut1 C-terminal tail is required for maximal growth factor-stimulated surface expression

Glut1 cell surface localization in haemopoietic cells is regulated by cytokines, such as IL-3, and activation of the PI3K (phosphoinositide 3-kinase)/Akt pathway [10]. To identify mechanisms that regulate Glut1 trafficking and are specific to Glut1, we compared the ability of cytokines to regulate the trafficking of Glut1 with the glucose transporter, Glut3. Glut3 is closely homologous with Glut1 yet divergent in the C-terminus, a domain previously implicated as important in Glut1 trafficking [15]. Cells were transfected with N-terminally GFP-tagged Glut1 or Glut3 fusion proteins and were imaged by confocal microscopy in the presence or after a 6 h withdrawal from IL-3 (Figure 1A). In contrast with GFP–Glut1, which showed cell surface levels regulated by IL-3 consistent with previous results [10], GFP–Glut3 was observed in an intracellular punctate pattern both in the presence and absence of IL-3 with very little GFP–Glut3 present near the cell surface.

The distinct C-terminal tails of Glut1 and Glut3 may have determined the different intracellular localization of these transporters and regulation by IL-3. To further establish the role of the Glut1 C-terminus in protein localization, surface levels of deletion or chimaeric Glut1 were analysed. A tandem FLAG epitope tag was inserted into the exofacial loop of wild-type and mutant Glut1 to allow flow cytometric measurement of surface Glut1 and trafficking without interference with any intracellular Glut1 domains [10]. FLAG-tagged deletion mutants of Glut1 were generated that lacked the last 38 amino acids of the cytoplasmic C-terminus (FLAG–Glut1ΔC) or lacked the C-terminal four amino acids (FLAG–Glut1Δ4) that constitute the PDZ-binding motif in Glut1. In addition, a FLAG-tagged chimaeric protein was made by replacing the C-terminal tail of Glut1 with the corresponding amino acids of Glut3 (FLAG–Glut1/CtermGlut3) (Figure 1B). Cells stably expressing FLAG-tagged chimaera and deletion Glut1 constructs were washed and cultured in the presence or absence of IL-3 for 6 h and surface levels were analysed by flow cytometry. As we have previously shown, full-length FLAG–Glut1 had high surface expression in the presence of IL-3 that decreased on growth factor withdrawal (Figure 1C and [10]). FLAG–Glut1ΔC and FLAG–Glut1Δ4 were found to have lower surface expression than full-length FLAG–Glut1 and these surface levels were further decreased on cytokine withdrawal. Replacement of the Glut1 C-terminal tail with that of Glut3 also reduced cell surface Glut1 expression to a level comparable with that of Glut1 ΔC (Figure 1C).

The Glut1 C-terminus may have affected Glut1 surface levels by altering Glut1 trafficking and/or protein levels. To address this, total cellular expression of wild-type Glut1, deletions and chimaeric Glut1 proteins was determined by immunoblotting (Figure 1D). Since slight differences of total FLAG–Glut1 expression levels in the presence of IL-3 were observed, surface FLAG–Glut1 levels were normalized to total Glut1 expression levels. The decreased surface levels of the truncation mutants or chimera in the presence of IL-3 reflected true changes in Glut1 distribution or trafficking when normalized to total levels (Figure 1E). Interestingly, while withdrawal from cytokine for 6 h did not substantially affect total FLAG–Glut1 levels, FLAG–Glut1 ΔC and FLAG–Glut1 Δ4 protein levels were sharply reduced upon cytokine withdrawal (Figure 1D), suggesting possible rapid targeting of internalized Glut1 deletion mutants for degradation.

Differences in protein levels of Glut1 deletion mutants were most pronounced when cells were deprived of growth factors. We have previously shown that Akt is a critical mediator of growth factor receptor signals to regulate Glut1 trafficking [10]. We tested, therefore, whether Akt was sufficient to rescue expression and prevent degradation of the FLAG–Glut1Δ4 mutant. FLAG–Glut1 and FLAG–Glut1Δ4 cells were transiently transfected with a constitutively active form of Akt [myrAkt (myristoylated Akt)] and cultured in the presence or absence of IL-3 for 6 h (Figures 2A and 2B). Activated Akt increased Glut1 protein levels and prevented the degradation of both full-length Glut1 and Glut1 Δ4 in the presence or absence of IL-3 (Figure 2A). In addition, activated Akt was sufficient to prevent the decrease in surface levels of FLAG–Glut1 Δ4 during growth factor withdrawal (Figure 2B). Expression of activated Akt could also protect Glut1 lacking the entire C-terminus from internalization and degradation (results not shown). Akt activation was not sufficient, however, to rescue the total and surface levels of the FLAG–Glut1 Δ4 mutant relative to the level of wild-type FLAG–Glut1. Therefore active Akt can maintain surface Glut1 in the absence of growth factor independent of the Glut1 C-terminus, possibly by inhibiting Glut1 internalization [10], but the PDZ-binding motif of Glut1 is required for both maximal total and surface levels.

Glut1 Lys-456 regulates Glut1 levels

In addition to the C-terminal four amino acids of Glut1, Ubc9 modification of Lys-456 of the Glut1 C-terminal tail has been proposed to regulate Glut1 protein levels [17]. To determine whether
Figure 1 The Glut1 C-terminal tail is required for maximal surface Glut1 localization

(A) FL5.12 cells were transiently transfected with GFP–Glut1 or GFP–Glut3 for 18 h and then cultured in the presence or absence of IL-3 for an additional 6 h. Cells were visualized by using confocal microscopy. (B) Schematic diagram of FLAG-tagged Glut1 full-length, C-terminal tail truncation (FLAG–Glut1ΔC), C-terminal four-amino-acid (4aa) truncation (FLAG–Glut1Δ4) and Glut1 chimaeric protein with C-terminus of Glut3 (Glut1/CtermGlut3). (C, D) Cells stably expressing FLAG–Glut1 constructs as shown in (B) were cultured in the presence or absence of IL-3 for 6 h. (C) Surface levels of FLAG–Glut1 were measured by flow cytometry. (D) Total FLAG–Glut1 levels were measured by immunoblotting (IB) with actin used as a loading control. (E) Cell surface FLAG–Glut1 levels were normalized to total FLAG expression. Representative results are shown for three or more experiments. Results are means ± S.D., with each experiment performed in triplicate. *P ≤ 0.001 within the experiment.

Lys-456 of the Glut1 C-terminal tail-regulated Glut1 degradation and whether it modulated the effect of the four-amino-acid truncation mutant, Lys-456 was mutated to alanine (FLAG–Glut1K456A) and a double mutant containing the K456A point mutation and truncation of the last four amino acids was generated (FLAG–Glut1K456A/Δ4). Cells stably expressing FLAG–Glut1K456A and FLAG–Glut1K456A/Δ4 were cultured in the presence or absence of IL-3 for 6 h, and total and surface FLAG–Glut1 levels were compared with both the full-length and four-amino-acid truncation mutant of FLAG–Glut1 (Figure 3A). The FLAG–Glut1K456A mutant exhibited a large increase in total FLAG–Glut1 expression levels when compared with full-length FLAG–Glut1, whereas FLAG–Glut1K456A/Δ4 had diminished surface levels similar to the four-amino-acid truncation mutant (Figure 3B). Although surface and total levels of Glut1 were increased on mutation of Lys-456, the glucose uptake of FLAG–Glut1K456A was similar to full-length Glut1, whereas the double mutant had comparable glucose uptake with the four-amino-acid truncation mutant (Figure 3C). Taken together, these results suggest that possible modification of Lys-456 may favour Glut1 degradation, but that the C-terminal four amino acids ultimately determine the fate of Glut1.

Targeting of Glut1 to lysosomes is regulated by the Glut1 C-terminal four amino acids

Degradation of internalized Glut1 on growth factor withdrawal probably occurred in lysosomes [11]. To test whether lysosomal...
Figure 2  Akt can prevent degradation of Glut1Δ4
(A) Total levels of FLAG–Glut1 and phospho-Akt (Ser-473) were measured by immunoblotting (IB) with actin used as a loading control. (B) Surface FLAG–Glut1 levels were measured by flow cytometry. Representative results are shown for three or more experiments. Results are means ± S.D., with each experiment performed in triplicate. * P < 0.001 within the experiment. myrAkt, myristoylated Akt.

Figure 3  Glut1 Lys-456 and the C-terminal PDZ-binding motif regulate Glut1 degradation
Cells stably expressing FLAG–Glut1, FLAG–Glut1Δ4, FLAG–Glut1K456A and FLAG–Glut1K456A/Δ4 were cultured in the presence or absence of IL-3 for 6 h. (A) Total FLAG expression levels were measured by immunoblotting (IB) with actin used as a loading control. (B) Surface FLAG levels were analysed by flow cytometry. (C) Glucose uptake was measured. Representative results are shown for three or more experiments. Results are means ± S.D., with each experiment performed in triplicate. * P < 0.005 and ** P < 0.01 within the experiment.
proteolysis of Glut1 was regulated by the C-terminal four amino acids of Glut1. N-terminal GFP-tagged Glut1 and Glut1Δ4 were analysed by fluorescence microscopy for co-localization with Lysotracker Red, a dye that stains lysosomes on the basis of low lysosomal pH level. Cells were transiently transfected with either GFP–Glut1 or GFP–Glut1Δ4 and cultured in the presence or absence of IL-3 for 8 h. To ensure that activation of cell death pathways did not alter protein trafficking during the growth factor withdrawal, cells were also transiently transfected with the anti-apoptotic protein, Bcl-XL. Glut1 remained on the cell surface in the presence of IL-3 with very little intracellular staining [10], however, intracellular Glut1 protein was observed to co-localize with lysosomes on growth factor withdrawal (Figure 4A). In contrast, the four-amino-acid truncation mutant of Glut1 resided...
mainly intracellularly and largely co-localized with lysosomes both in the presence and absence of the cytokine IL-3. These results suggest that the C-terminal four amino acids may be critical to protect Glut1 from lysosomal targeting following growth factor deprivation.

The role of lysosomes in degradation of Glut1Δ4 was further analysed using pharmacological and genetic techniques. Chloroquine and ammonium chloride can neutralize lysosomal pH to inhibit lysosomal proteolytic capacity [25–27]. FLAG–Glut1- and FLAG–Glut1Δ4-expressing cells were cultured in the presence or absence of IL-3 for 6 h with vehicle control, chloroquine or ammonium chloride for the duration of the growth factor withdrawal (Figure 4B). Chemical inhibition of lysosomes did not alter full-length FLAG–Glut1 total protein levels in the presence or absence of cytokine IL-3 for the time points analysed here. An increase in protein levels, however, was observed in the Glut1 four-amino-acid truncation mutant when lysosomal degradation was inhibited in the presence or absence of IL-3. A genetic approach was also employed to confirm a role for lysosomal targeting in the degradation of Glut1Δ4. The small GTPase Rab7 has been implicated in targeting proteins for lysosomal degradation [11,28–30]; therefore we utilized an shRNAi construct containing a previously published and validated target sequence to down-regulate Rab7 expression [11]. Control shRNAi or Rab7 shRNAi constructs were transiently transfected into FLAG–Glut1 and FLAG–Glut1Δ4 cells to reduce Rab7 expression and cells were subjected to a 6 h growth factor withdrawal (Figure 4C). Similar to chemical inhibition of lysosomes, Rab7 deficiency largely prevented the degradation of FLAG–Glut1Δ4. Neither pharmacological or genetic approaches to block lysosomal degradation fully rescued FLAG–Glut1Δ4 protein levels, thereby indicating that each approach was not fully efficient or that alternative proteolytic pathways may also regulate Glut1. Taken together, these results show that Glut1 degradation by lysosomes is regulated by the last four amino acids, DSQV, which are critical to protect intracellular Glut1 from lysosomal degradation.

Amino acids dictating PDZ-binding specificity regulate Glut1 total and surface levels

It has been demonstrated that individual amino acids within a class I PDZ-binding motif are critical in determining what protein–protein interactions occur and a given PDZ-binding motif may interact with a number of PDZ-domain-containing proteins. In particular, the binding specificity of class I PDZ-binding motifs is dependent on C-terminal amino acids in the 0 and –2 positions [31]. To further characterize the role of the Glut1 PDZ-binding motif, each amino acid was mutated and total and surface levels of FLAG–Glut1 were observed (Figures 5A and 5B). Mutation of all four amino acids to alanine residues resulted in a phenotype similar to the four-amino-acid truncation mutant with low total and surface levels. Point mutation of the –2 (DAQV) and –3 (ASQV) positions did not impact total FLAG–Glut1 levels but caused a slight decrease in surface FLAG–Glut1 levels. In contrast, point mutation of the 0 (DSQA), –1 (DSAV) or 0 and –2 (DAQA) positions each significantly decreased total protein levels and surface levels. Even though total levels of the –1 point mutation (DSAV) were almost undetectable by immunoblotting, surface levels of FLAG–Glut1-DSAV were detected at levels higher than background staining control by flow cytometry. While Q to A (–1) mutations represent a charge change that may, in principle, affect protein folding, the V to A (0) mutation is structurally well conserved but also led to a loss of Glut1 protein. These results support a necessary role for the Glut1 PDZ-binding domain and possible interaction with class I PDZ-domain proteins as essential for regulation of Glut1 trafficking.

Glut1 levels may be regulated through control of either Glut1 internalization or targeting to lysosomes, and mutations in the Glut1 PDZ-binding domain may have affected either or both processes. To test whether this PDZ-binding domain affected Glut1 internalization, a pulse–chase assay was performed to measure the internalization rate of full-length FLAG–Glut1, FLAG–Glut1Δ4 and FLAG–Glut1-DSQA in the presence or absence of growth factor (Figure 5C). Cells were stained with anti-FLAG primary antibody and placed at 37°C for the times indicated prior to addition of fluorescently labelled secondary antibody. Flow cytometry was used to measure cell fluorescence and to determine the fraction of FLAG-tagged Glut1 and primary antibody remaining on the cell surface at each time point. As we have previously shown, internalization of full-length Glut1 was more rapid in growth factor-deprived cells than in the presence of growth factor [10]. Glut1 lacking the C-terminal four amino acids and Glut1-DSQA both internalized more rapidly than full-length Glut1 in the presence of growth factor and had an even more rapid loss of cell surface levels in the absence of growth factor. Although the Glut1 PDZ-binding motif may play multiple roles in Glut1 trafficking, these results suggest that this domain contributes to regulation of Glut1 internalization.

The PDZ-domain protein GIPC has been described to associate with the C-terminus of Glut1 and these interactions may mediate the effects of the Glut1 PDZ-binding domain in Glut1 trafficking. To determine whether GIPC associated with full-length Glut1, but not PDZ-domain mutants of Glut1, His6-tagged GIPC was purified and used to coat nickel beads (Figure 5D). Cells were transiently transfected to express FLAG-tagged Glut1, Glut1Δ4 or Glut1 point mutations DSQA, DAQV, DSAV or ASQV and cell lysates were mixed with GIPC-labelled beads or unlabelled beads for pulldown analyses (Figure 5D). As expected, full-length Glut1 bound GIPC-labelled beads, but not unlabelled beads. Glut1Δ4 or Glut1-DSQA also showed binding to GIPC, although in each case binding was reduced compared with that of full-length Glut1 (note the quantifications of the ratio of input Glut1 to GIPC-bound Glut1 indicated below each band). As shown above, Glut1 DSQV was not readily detectable and could not be quantified, but the Glut1 DAQV and ASQV mutants each bound GIPC with similar or greater efficiency than wild-type Glut1. Thus, although the Glut1 PDZ-binding domain does not appear to be the sole determinant of Glut1 association with GIPC, it does provide a significant contribution to this interaction.

GIPC does not regulate Glut1 degradation or internalization but is required for efficient surface Glut1 localization

Based on the critical role of the Glut1 PDZ-binding domain in regulation of Glut1 lysosomal degradation and the association of GIPC with this domain (Figure 5D) [16,23], we sought to determine whether GIPC regulated Glut1 and was necessary to protect Glut1 from lysosomal degradation. Two individual shRNAi constructs were generated to target GIPC and decrease GIPC expression. Glucose uptake was significantly reduced in GIPC-deficient cells in the presence of IL-3 relative to cells expressing a control shRNAi construct (Figure 6A). A more modest yet significant difference was observed in IL-3-withdrawn cells. To determine whether GIPC was necessary to maintain Glut1 protein levels and prevent Glut1 lysosomal targeting, total levels of endogenous Glut1 were measured by immunoblotting (Figure 6B). In contrast with the loss of Glut1 protein observed by deletion of the C-terminal-binding motif of Glut1, no loss of total Glut1 protein was detected when GIPC was down-regulated.
Figure 5  The Glut1 PDZ-binding motif is critical in the regulation of Glut1 total and surface levels

(A–C) Cells were transiently transfected with FLAG–Glut1 constructs containing point mutations in the PDZ-binding motif, as noted in bold, for 18 h and then cells were cultured in the presence or absence of IL-3 for an additional 6 h. (A) Total FLAG–Glut1 was measured by immunoblotting (IB) with actin used as a loading control. (B) Flow cytometric analysis was utilized to measure surface FLAG–Glut1 levels. FL5.12 cells without FLAG–Glut1 were used as a negative stain control. (C) Cells were stained with anti-FLAG antibody, washed and cultured at 37°C for the indicated times prior to staining with fluorescent secondary antibody to determine the fraction of surface label remaining after various incubation times. (D) Cells were transiently transfected with FLAG–Glut1, FLAG–Glut1Δ4 and FLAG–Glut1 point mutants (DSQA, DAQV, DSAV and ASQV) constructs for 24 h and lysates were prepared. Lysates were then incubated with purified uncoated or nickel-bound His6–GIPC beads. Anti-6×His immunoblotting (IB) compared uncoated beads with nickel-bound His6–GIPC beads used for pulldown. After washing, beads were boiled with SDS sample buffer and FLAG levels were measured via immunoblotting. G, nickel-bound His6–GIPC beads; I, input cell lysate; ND, not determined; P, pulldown with His6–GIPC beads; U, uncoated beads. Representative results are shown for three or more experiments. Results are means ± S.D., with each experiment performed in triplicate. *P < 0.0005 and **P ≤ 0.005 within the experiment.

These results demonstrate that GIPC association with the Glut1 C-terminal PDZ-binding motif is required to promote maximal glucose uptake, but is not required to protect Glut1 from lysosomal degradation.

The decreased glucose uptake but maintenance of Glut1 protein levels in GIPC-deficient cells suggested that the Glut1 C-terminus may interact with GIPC to regulate non-lysosomal trafficking of Glut1. To determine whether Glut1 trafficking was affected by GIPC deficiency, surface expression of FLAG–Glut1 was determined in control and GIPC-deficient cells in the presence of IL-3 or after a 6 h IL-3 withdrawal, and normalized to total FLAG–Glut1 levels (Figures 6C and 6D). GIPC deficiency led
Glut1 PDZ motif determines the fate of Glut1

Figure 6  GIPC deficiency decreases glucose uptake and surface Glut1 levels, but does not cause Glut1 degradation

(A, B) FL5.12 cells were transiently transfected with either control shRNAi, GIPC shRNAi1 or GIPC shRNAi2 constructs for 48 h and were then cultured in the presence or absence of IL-3 for 6 h. (A) Glucose uptake was measured. (B) Total endogenous Glut1 and GIPC levels were measured by immunoblotting (IB) with actin used as a loading control. (C–E) FLAG–Glut1- and FLAG–Glut1Δ4-expressing cells were transiently transfected with control shRNAi, GIPC shRNAi1 or GIPC shRNAi2 constructs for 48 h followed by culture in the presence or absence of IL-3 for 6 h. (C) Total FLAG–Glut1 and GIPC levels were measured by immunoblotting (IB) with actin used as a loading control. (D) Total surface FLAG–Glut1 levels were measured by flow cytometry and surface levels were normalized to total FLAG expression levels. (E) Surface FLAG–Glut1Δ4 levels were measured using flow cytometry. (F) FLAG–Glut1 cells were transfected with control shRNAi or GIPC shRNAi1 constructs for 48 h and cultured in the presence of IL-3. Cells were stained with anti-FLAG antibody, washed and cultured at 37°C for indicated times prior to staining with fluorescent secondary antibody. Representative results are shown for three or more experiments. Results are means ± S.D., with each experiment performed in triplicate. *P < 0.05 and **P < 0.01 within the experiment.

to markedly decreased surface FLAG–Glut1 in the presence of IL-3 that decreased further after IL-3 withdrawal (Figure 6D). Importantly, loss of surface FLAG–Glut1 in GIPC-deficient cells did not reflect a loss of Glut1 protein as FLAG–Glut1 levels were the same or modestly increased in the absence of GIPC (Figure 6C). This decrease in surface FLAG–Glut1 relative to control RNAi was observed with two independent RNAi constructs targeting GIPC (Figure 6D). In addition, decreased surface FLAG–Glut1 levels caused by GIPC deficiency were specific to full-length FLAG–Glut1 as surface levels of Glut1 lacking the C-terminal four amino acids critical for interaction with GIPC were unaffected by loss of GIPC (Figure 6E).
 Reduced surface levels of Glut1 may have been due to more rapid internalization or failure of intracellular Glut1 to recycle back to the cell surface. Having shown that Glut1 lacking four amino acids or with mutation in the C-terminus has more rapid internalization (Figure 5C), we tested the role of GIPC in Glut1 internalization. A pulse–chase assay for surface Glut1 internalization was performed on cells transfected with control or GIPC shRNAi (Figure 6F). GIPC deficiency did not alter the internalization rate of Glut1 compared with cells transfected with control RNAi. We have previously shown that recycling of Glut1 is essential for maximal surface levels [10]. The decreased surface Glut1 levels, normal Glut1 internalization and normal total cellular levels of Glut1 suggest that GIPC may be essential for trafficking of intracellular Glut1 back to the cell surface.

Disruption of GIPC alters Glut1 subcellular localization and suggests a Glut1 recycling pool

GIPC deficiency decreased surface FLAG–Glut1 leading to accumulation of intracellular Glut1. To characterize how GIPC affected Glut1 intracellular trafficking and this pool of intracellular Glut1, we transiently transfected cells with GFP–Glut1 and control shRNAi or GIPC shRNAi1 constructs for 48 h in the presence of IL-3. Lysotracker Red was added 30 min prior to fixation and cells were visualized with fluorescence microscopy to compare GFP–Glut1 localization with lysosomes (Figure 7A). Partial co-localization of GFP–Glut1 with lysosomes was observed in both control shRNAi and GIPC shRNAi1 cells; however, most of the GFP–Glut1 resided on the cell surface in control cells, whereas in GIPC-deficient cells, GFP–Glut1 resided in intracellular compartments that were distinct from lysosomes. These results suggested that GIPC deficiency may lead to Glut1 accumulation in the endosomal recycling pool. Given that this recycling pathway is commonly associated with trafficking of the TfR, we investigated whether GFP–Glut1 co-localized with TfR in control and GIPC-deficient cells (Figure 7B). Cells were transfected with GFP–Glut1 and treated with fluorescently labelled transferrin to label TfR and its trafficking patterns. Importantly, GFP–Glut1 did not co-localize with transferrin in either control or GIPC-deficient cells, suggesting that Glut1 exists in an independent or specialized

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**Figure 7** Disruption of GIPC suggests that Glut1 can exist in a recycling pool distinct from TfR

(A, B) FL5.12 cells were transfected with GFP–Glut1 and control shRNAi or GIPC shRNAi1 constructs for 48 h and were incubated with (A) 50 nM Lysotracker Red for 30 min or (B) 50 μg/ml Alexa Fluor® 568-tagged transferrin for 1 h followed by fixation and visualization with fluorescence microscopy. (C) Cells were transfected with control shRNAi or GIPC shRNAi constructs for 48 h and were then cultured in the presence or absence of IL-3 for an additional 6 h. Cells were stained with anti-TfR antibody and surface levels of TfR were measured via flow cytometry. Representative results are shown for three or more experiments. Results are means ± S.D., with each experiment performed in triplicate. *P < 0.0005 and **P ≤ 0.05 within the experiment.
Representative results are shown for three or more experiments. Results are means +− S.D., with each experiment performed in triplicate. *P < 0.005 within the experiment.

Figure 8  Inhibition of Glut1 degradation does not lead to high surface Glut1 levels but allows a portion of Glut1 to rapidly recycle following growth factor stimulation

(A) FLAG–Glut1 cells were transiently transfected with control shRNAi or Rab7 shRNAi plasmids for 24 h and cultured for an additional 6 h in the presence or absence of IL-3. Surface FLAG–Glut1 levels were analysed by flow cytometry. (B) FLAG–Glut1 cells stably expressing hBcl-X₇ were transfected with control shRNAi or Rab7 shRNAi for 24 h followed by a 24 h growth factor withdrawal. Surface FLAG–Glut1 levels were measured by flow cytometry after 0, 15, 30 and 60 min of IL-3 re-addition. (C) FLAG–Glut1 cells stably expressing hBcl-X₇ were withdrawn from IL-3 for 24 h with either vehicle or 40 μM chloroquine added during the last 6 h of starvation before re-addition of IL-3. Surface FLAG–Glut1 levels were measured by flow cytometry 15 min after re-addition of IL-3. Representative results are shown for three or more experiments. Results are means ± S.D., with each experiment performed in triplicate. *P < 0.005 within the experiment.

Prevention of Glut1 degradation allows more rapid recovery of cell surface Glut1 levels

It was unclear how intracellular accumulation of Glut1 and inhibition of Glut1 degradation following growth factor withdrawal would affect surface Glut1 levels. In principle, accumulation of excess intracellular Glut1 may lead to growth factor-independent return of Glut1 to the cell surface. Alternatively, excess Glut1 may remain intracellular, but allow enhanced recovery of surface Glut1 levels on restimulation. To test this, we transfected cells with control or Rab7 shRNAi to prevent Glut1 lysosomal targeting and measured surface levels of FLAG–Glut1 in the presence or absence of IL-3 for 6 h (Figure 8A). At this early time point, inhibiting lysosomal targeting of Glut1 by Rab7 deficiency modestly increased surface FLAG–Glut1 levels compared with control cells. To analyse how prevention of Glut1 lysosomal degradation affected Glut1 trafficking at later time points, Bcl-X₇ was expressed to prevent cell death and cells were growth factor withdrawn for 1 day prior to analysis. After 1 day without growth factor, surface Glut1 levels were greatly diminished regardless of control shRNAi or Rab7 shRNAi or chloroquine treatment, although inhibition of Glut1 degradation did provide a small increase in surface Glut1 levels (Figures 8B and 8C, note the scales). Importantly, a portion of this intracellular Glut1 was capable of rapid return to the cell surface after growth factor restimulation of both Rab7 shRNAi-transfected and chloroquine-treated cells. Increased intracellular Glut1 levels, therefore, both enhance growth factor-independent surface Glut1 levels and allow more rapid recovery of surface Glut1 after growth factor stimulation.

DISCUSSION

In haemopoietic cells, growth factors and cytokines regulate not only Glut1 synthesis [13,32,33], but also control Glut1 trafficking to and from the cell surface [10] to regulate glucose uptake. It has been suggested that internalized Glut1 is ultimately recycled back to the cell surface or degraded in lysosomes [11,23]. Little is known, however, about this process and direct evidence for Glut1 degradation and regulation of this degradation has been lacking. We show in the present study that the Glut1 C-terminal tail is critical for determining the fate of internalized Glut1 to either accumulate intracellularly or be degraded by lysosomes. Utilizing truncation and point mutants of Glut1, we demonstrated that the C-terminal four- amino-acid PDZ-binding domain of Glut1 is necessary for maximal surface localization and to avoid lysosomal degradation. A PDZ-domain protein, GIPC, could bind this C-terminal motif in Glut1 and was necessary for efficient return of internalized Glut1 back to the cell surface as GIPC deficiency resulted in accumulation of an intracellular pool of Glut1. We suggest, therefore, that the C-terminal four amino acids of Glut1 serve as a critical platform for protein–protein interactions that regulate multiple aspects of Glut1 trafficking and may control levels of intracellular Glut1 that may return to the cell surface on restimulation.

Growth factor regulation of Glut1 localization relies on proper control of Glut1 internalization, recycling and lysosomal targeting. The balance and how growth factors, such as IL-3, influence each of these processes, however, have been unclear. In addition to previous work showing that IL-3 and Akt can inhibit Glut1 internalization, we show here that inhibition of endosomal recycling by shRNAi of GIPC or expression of dominant-negative Rab11a [10] strongly decreased surface Glut1 levels in growth factor-stimulated cells. These results suggest an important role for recycling of Glut1 in supporting growth factor-stimulated glucose uptake. Growth factors were also found to suppress Glut1 targeting to lysosomes, as lysosomal degradation of Glut1 appeared to increase in the absence of growth factor, particularly if the C-terminal PDZ-binding domain of Glut1 was mutated. Thus growth factor signalling appears to attenuate Glut1 internalization,
promote Glut1 recycling and inhibit Glut1 degradation. The signalling mechanisms mediating this growth factor regulation of Glut1 trafficking are not clear. Akt activation can prevent Glut1 internalization but did not fully rescue protein levels of Glut1 lacking the PDZ-binding domain. PDZ-domain interactions with Glut1, therefore, appear likely to regulate Glut1 levels and glucose uptake independent of Akt.

Despite co-ordinated growth factor control, Glut1 recycling and lysosomal targeting were not directly linked and inhibition of either process led to accumulation of intracellular Glut1 rather than to the opposite fate. Inhibition of Glut1 lysosomal targeting led largely to accumulation of intracellular Glut1 rather than recycling of Glut1 back to the cell surface. Similarly, loss of GIPC led to accumulation of intracellular Glut1 rather than targeting of Glut1 to lysosomes. This intracellular pool of Glut1 was independent of the common TIR recycling pool and may represent a novel Glut1 trafficking mechanism. Importantly, a portion of this intracellular Glut1 could rapidly return to the cell surface following growth factor stimulation. That only a portion of Glut1 could rapidly recycle back to the cell surface after growth factor stimulation may indicate that much of the Glut1 was trafficked to a compartment incapable of rapid recycling. Alternatively, the protein machinery necessary for rapid recycling may be diminished after a 1 day growth factor withdrawal. These findings contrast somewhat with previous studies that have shown Rab7 deficiency to prevent targeting of nutrient transporters to lysosomes, which allowed maintenance of nutrient uptake via recycling of transporters back to the cell surface even in the absence of growth factors [11]. It is possible, however, that Glut1 is regulated differently than other nutrient transporters through specific interaction with multiple PDZ-domain proteins such as GIPC. In support of this notion are findings that Glut1 trafficking is rapamycin-insensitive [10], while other nutrient transporters are rapamycin-sensitive [34]. Alternatively, deficiency in lysosomal targeting via Rab7 may affect other cellular processes that can sustain cell metabolism and viability independent of Glut1.

Mutation of the Glut1 C-terminal four-amino-acid PDZ-binding motif had a profound effect on Glut1 and resulted in decreased total and surface Glut1 levels. Interactions with PDZ-domain-containing proteins, therefore, are likely to be critical in the regulation of Glut1. The PDZ-domain-mediated interaction between Glut1 and GIPC appeared to play a key role in regulation of Glut1 recycling. The intracellular punctate localization close to the plasma membrane and association of GIPC with cytoskeletal elements, such as Myo6 and actin [23,35,36], have previously suggested that GIPC may play a role in vesicle sorting to the cell surface. Consistent with this proposed role for GIPC, we found increased intracellular levels of Glut1 in GIPC-deficient cells, particularly in the presence of growth factor. It is not clear, however, whether or how GIPC may be regulated by growth factor to promote Glut1 recycling. Given the role of GIPC in trafficking of other cell surface receptors to the plasma membrane [20–22], it will be important in future studies to determine whether growth factor signalling pathways may also affect their recycling via regulation of GIPC.

In addition to recycling, Glut1 also required the PDZ-binding domain to be protected from rapid targeting to lysosomes for degradation after growth factor withdrawal. Deficiency of GIPC, however, did not lead to Glut1 degradation similar to that observed in Glut1 lacking the PDZ-binding motif or with mutation of the C-terminal valine, a residue known to be important in the specificity of PDZ binding [31]. These results suggest that an additional protein may interact with the Glut1 PDZ-binding motif and may associate with Glut1 to prevent Glut1 lysosomal degradation. Reminiscent of this model is the β2-adrenergic receptor, which can interact with multiple PDZ-domain proteins and requires association with the PDZ-domain protein, EBPs50, to prevent β2-adrenergic receptor targeting to lysosomes for destruction [37]. It remains unclear, however, how the Glut1 PDZ-binding motif may protect intracellular Glut1 from lysosomal targeting and degradation.

SUMOylation of Lys-456 of the Glut1 C-terminal tail by Ubc9 has also been shown to regulate total Glut1 protein levels [17]. Mutation of Lys-456 to alanine altered Glut1 levels, with accumulation of both surface and total Glut1 that contrasted starkly with the loss of Glut1 protein observed with mutation of the PDZ-binding domain. Importantly, mutation of both critical sites in the Glut1 C-terminus (K456A/D4) presented with the phenotype of the PDZ-binding domain mutant. The Glut1 PDZ-binding motif and possible interactions with PDZ-domain proteins, therefore, represent the dominant determinant in the fate of Glut1. It may be that Glut1 interaction with a PDZ protein is affected by lysine modification. This type of regulation has been described for the transcriptional co-repressor, CtBP, which is regulated by antagonistic PDZ protein binding and sumoylation [38], and may also occur for Glut1.

In the present study, we demonstrate that the Glut1 C-terminal four amino acids are a key component in growth factor regulation of Glut1. Interestingly, this PDZ-binding motif was essential to allow existence of intracellular Glut1 after growth factor withdrawal, a portion of which could be rapidly returned to the cell surface on restimulation. Similar to Glut4, which is stored intracellularly in specialized storage vesicles [39,40], Glut1 may also exist in intracellular pools that are independent of standard endosomal and recycling pathways that encompass TIR recycling and dependent on GIPC to return to the cell surface. This trafficking pattern would be less dynamic than that observed in insulin-regulated Glut4 trafficking, but nevertheless provides a mechanism to rapidly retrieve some Glut1 to the cell surface that should a growth signal be received. Taken together, these results show that Glut1 is highly regulated through multiple modifications or interactions. As Glut1 is a central glucose transporter in many tissues, these points of Glut1 regulation are important for further study as they may impact cellular glucose homeostasis in both normal and pathological states.

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