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# Molecular characterization of a glycerophosphoinositol transporter in mammalian cells

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**Abstract** **The glycerophosphoinositols are ubiquitous phosphoinositide metabolites involved in the control of several cell functions. They exert their actions both intracellularly and by rapidly equilibrating across the plasma membrane when added to cells, implying the existence of a transporter for their membrane permeation. Such a transporter, *GIT1*, has been cloned in yeast. By PSI-BLAST analysis, we have identified the Glut2 transporter as a human-genome candidate ortholog of *GIT1*. This was supported directly through the use of inhibitors, siRNAs and competition studies of specific uptake of GroPIns in HeLa cells over-expressing human Glut2. These data identify Glut2 as a GroPIns transporter in mammals, and define a physiologically relevant cell-permeation mechanism.**


*Keywords:* GIT1; Phosphoinositide metabolism; Cell regulation; Inositol-containing molecules; Actin cytoskeleton; Tumor invasion

## 1. Introduction

The glycerophosphoinositols are intracellular, water-soluble phosphoinositide metabolites that are involved in the control of signalling enzymes (e.g. adenylyl cyclase [1]), in actin cytoskeleton organization [2], in invasion of the extracellular matrix by melanoma and breast carcinoma cell lines [3], and in the control of cell proliferation in epithelial cells [4,5]. Of these compounds, the most abundant in cells is glycerophosphoinositol (GroPIns), with its intracellular concentrations generally ranging from 50 to 450 μM across different normal and transformed cell lines [5,6]. The intracellular accumulation of this compound was originally correlated to the activation of the Ras pathway [7,8] and to the differentiation/dedifferentiation of several cell lines, including macrophages and hepatocytes [9–12].

In yeast, GroPIns is secreted as a product of phosphoinositide catabolism, and can be taken up from the extracellular medium and incorporated into the phosphoinositides when the inositol supply is scarce or in phosphate-starved yeast cells [13–17]. GroPIns is transported across the yeast plasma membrane by a well-characterized mechanism that involves a permease encoded by the 1556-bp *GIT1* gene, which codes for a 518-amino-acid protein with a predicted molecular mass of 57.3 kDa [16,17]. Gitlp contains the SDRIGR(K/R) sugar-transport motif around amino acid 329 and 12 potential membrane-spanning domains. Specificity studies have indicated that GroPIns transport in yeast is not affected by excess glycerol or inositol, is inhibited by excess glycerol 3-phosphate, and is only partially inhibited by excess glycerophosphocholine and glycerophosphoethanolamine [16]. *GIT1* expression and Gitlp transport activity are induced in response to inositol deprivation and phosphate deprivation [17]. The combination of Gitlp and extracellular phospholipase activity, therefore, provide yeast with the ability to obtain crucial nutrients (inositol and phosphate) in environments in which phosphatidylinositol or GroPIns are available. Although a similar mechanism has been proposed for glycerophosphoinositol transport in mammalian cells [2,18], no further information as to the molecular identity or activity of this transporter has been provided to date.

It has previously been shown that GroPIns is present in both intracellular and extracellular spaces [5,19], and that extracellular GroPIns is a substrate of specific phosphodiesterases that are able to hydrolyze it to inositol and glycerol phosphate [20]. As the phospholipase $A_2$ and the lysolipase that are involved in the formation of GroPIns are intracellular enzymes, our hypothesis is that a specific transporter exists in mammals that mediates both GroPIns uptake and exclusion, depending on the GroPIns concentration gradient across the plasma membrane. Under physiological conditions, this gradient could arise from the intracellular formation of GroPIns following phospholipase $A_2$ activation [4,5]; alternatively, it could derive from the exogenous application of GroPIns [2,3].

The understanding of the mechanism of transport of GroPIns across the plasma membrane is thus relevant to the full definition of its metabolism and mechanism of action. With the aim of identifying the GroPIns transporter, we carried out sequence analyses and biochemical studies, which have led to the identification of the permease Glut2 as the first GroPIns-specific transporter in mammalian cells.

## 2. Materials and methods

### 2.1. Materials

Lipofectamine-plus reagent was from Invitrogen (Ontario, Canada). Tetanolysin from *Clostridium tetani*, phloretin, fetal bovine serum

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(FBS), bovine serum albumin (BSA), D(+)glucosamine, $HgCl_2$ and the actin polyclonal antibody were from Sigma (MO, USA). [$^3$H] glucosamine (196 Gbq/mmol, 5.3 Ci/mmol) was from Amersham Biosciences (Buckinghamshire, UK). [$^3$H] GroPIns was prepared from [$^3$H] L-α-phosphatidylinositol (Perkin–Elmer, Boston, USA), (314.5 Gbq/mmol, 8.5 Ci/mmol) according to the deacylation procedure originally reported by Clarke and Dawson [21]. GroPIns was from Euticals S.p.A. (Lodi, Italy). All other reagents were of the highest purities available and were obtained from GIBCO Brl (Grand Island, NY).

### 2.2. Sequence analysis

The Gitlp sequence (accession number P25346) was compared with mammalian sequences using PSI-BLAST. The first iteration of PSI-BLAST showed an *e*-value of 0.027, with an 18% identity and a 33% similarity in an alignment comprising amino acids 84–475 of yeast Gitlp. The global alignment of yeast Gitlp and human Glut2 (hGlut2) was done with Clustal W, with a 13% score identity. The search of the phylogenetic patterns via the identification of common Eukaryotic Orthologous Groups (KOGs) was carried out with the NCBI CDD (Conserved Domain Database), and showed that yeast Gitlp and hGlut2 aligned significantly with: (i) the permease of the major facilitator superfamily (KOG0569), with *e*-values of $2\times e^{-7}$ and $1\times e^{-125}$, respectively; (ii) the predicted transporter (major facilitator superfamily) (KOG0254), with *e*-values of $5\times e^{-15}$ and $4\times e^{-60}$, respectively; (iii) the synaptic vesicle transporter SVOP and related transporters (major facilitator superfamily) (KOG0255), with *e*-values of $1\times e^{-7}$ and $1\times e^{-18}$, respectively; and (iv) the inorganic phosphate transporter (KOG0252), with *e*-values of $2\times e^{-130}$ and $2\times e^{-12}$, respectively.

### 2.3. Cell culture and transfection

HeLa cells were transfected with 8 μg cDNA/petri dish using the Lipofectamine-plus method, according to the manufacturer instructions. Human Glut2 transporter cDNA (1760 bp), generously provided by G.I. Bell (Howard Hughes Medical Institute, Chicago, USA), was subcloned in the pCMV4 expression vector (Invitrogen, CA, USA). The transfection efficiency, evaluated by immunofluorescence analysis (see below), was ~20%. Cytosol and membrane preparations were obtained according to Garcia-Higueras et al. [22]. Glut2 expression was verified by Western blotting, using rabbit antiserum against hGlut2 (1:1000) (Alpha Diagnostic, TX, USA), and by immunofluorescence analysis, with the same antiserum (1:100), as previously reported [2].

### 2.4. GroPIns uptake assay

HeLa cells were scraped from the petri dishes, and a fraction was used for counting and for a trypan blue vitality test. Vitality was always more than 95%. Two $\times 10^6$ cells/sample were resuspended in 150 μl buffer A (cell medium in which the cells had grown for at least 4 h, plus 25 mM HEPES, pH 7.4). The cellular uptake assays were initiated by adding this to 150 μl buffer A containing GroPIns or glucosamine, to give the final concentrations indicated, and the [$^3$H] GroPIns (8.5 Ci/mmol; ~400 000 cpm) or [$^3$H] glucosamine (5.3 Ci/mmol ~40 000 cpm) tracers, as appropriate. Where necessary, the osmolarity was corrected by inclusion of NaCl. The cells were then either filtered directly (see below; for the time zero background) or incubated for the times indicated in a 37 °C water bath under gentle shaking. The uptake was terminated by applying the cells onto nitrocellulose filters (Whatman, NJ, USA) on a vacuum-filter apparatus, followed by 3 × 1 ml washes with ice-cold buffer B (unlabelled GroPIns or glucosamine at the same concentration used during the uptake assays, plus 0.3% BSA, 1 mM $HgCl_2$ in PBS, pH 7.4); the filters were then dissolved in Filter Count scintillant (Perkin–Elmer) and counted.

Cell lysates were obtained using four cycles of freezing in liquid $N_2$ and thawing at 37 °C in a water bath sonicator. Tetanolysin permeabilization was performed with the procedure adapted from Riese et al. [23].

### 2.5. Glut2 RNAi

Glut2 sequence-specific silencing was performed in HEK293 cells with Glut2 siRNA(h) duplexes (Santa Cruz Biotechnology Inc., San Diego, CA, USA) and the siCONTROL non-targeting duplexes (Dharmacon Inc., Lafayette, CO, USA) using the Oligofectamine Reagent (Invitrogen) according to the manufacturer instructions. After silencing, the cells were seeded in serum-free growth medium for 4 h, then 10% FBS was added for a further 48 h before the uptake assays and Western blotting.

### 2.6. Statistical analysis

The data are expressed as means ± S.E. of three or four independent experiments, with each performed in quadruplicate. Statistical analysis was by Student's *t*-test.

## 3. Results

### 3.1. Identification of a Gitlp human ortholog

The initial analysis of Gitlp, the GroPIns transporter of *Saccharomyces cerevisiae*, and the sequence comparison with mammalian sequences by PSI-BLAST led to the identification of Glut2 as the closest human hit. Although the global alignment of the two sequences showed an identity of only 13%, a search for clusters of orthologous groups (see Section 2) clearly indicated that they belong to common clusters and that some of the amino acids essential for the transporter functions are conserved in both sequences (see Fig. 1).

Gitlp and Glut2 also share a similar structure, with sequence analysis studies predicting that they include 12 membrane-spanning helices (as underlined in Fig. 1; [16,24]). In addition, both proteins include the R(K/R)XGRR(K/R) motif in their sequence, which is recognized as being specific for the major facilitator superfamily (MFS) members (blue in Fig. 1; [16,24]).

The residues critical for the helix structure, including the glycines of the transmembrane regions that are characteristic of the Glut family, have been shown to be required for transporter function [24], and they are conserved in the Gitlp sequence (Fig. 1). Other important motifs of the Glut transporters that have been suggested to participate in substrate recognition, such as the STSIF-motif in loop 7, were also found in the Gitlp sequence (Fig. 1), whereas the QLS-motif (QFS for hGlut2) was not present.

Altogether these data point to a number of similarities between the Gitlp and Glut2 proteins, which led us to further investigate the transporter function of Glut2.

### 3.2. Glut2 over-expression in HeLa cells

To determine whether hGlut2 represents the mammalian counterpart of yeast Gitlp, we measured the uptake of GroPIns in HeLa cells over-expressing hGlut2. The mammalian expression vector carrying hGlut2 cDNA (hGlut2/pCMV4), or the vector alone (pCMV4, as mock), were transfected into HeLa cells, which do not endogenously express this transporter, and 24 h later the cells were used in the GroPIns uptake experiments. Human Glut2 over-expression and its membrane localization were confirmed by Western blotting of cell lysates (Fig. 2A, left panel); two major bands of ~57 and 59 kDa were specifically identified in *GLUT2*-transfected HeLa cells. Both bands correspond to the Glut2 transporter, but with different levels of glycosylation or, possibly, other post-translational modifications [25]. A third minor band at a lower molecular weight (~53 kDa) was recognized by the specific antibody; this can be assigned to the degradation products of Glut2 as it was detected only in transfected cells. Imm-

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Git1p   84   KVSTRVSNAA---LVGIIFGQFFMGIAADYYSRKSCILVATAILVIGSALCAAS-----H  135
              ++   S +     VG +   FF G   D   R   +LVA  + ++G+ L   S     H
hGlut2  92   LITMLWSLSVSSFAVGGMTASFFGGWLGDTLGRIKAMLVANILSLVGALLMGFSKLGPSH  151

Git1p  136   GTTVPGMFWMLT---VMRGLVGIGVGAEYPTSTLSANESANEYTTTKRGGILVMVTNLPL  192
                 + G         ++ GLV + +G   PT+   A  + ++      G ++  +  L
hGlut2 152   ILIIAGRSISGLYCGLISGLVPMYIGEIAPTALRGALGTFHQ-LAIVTGILISQIIGLEF  210

Git1p  193   AFGGPFATIIFLIVYKICSGTKHLEAIWRTVFAIGCFWPLSVFYFRWKTATTEVYEKGRI  252
                G      I L          + AI +++    C  P S  Y   K    EV  K  +
hGlut2 211   ILGNYDLWHILLG-------LSGVRAILQSLLLFFC--PESPRYLYIKLD-EEVKAKQSL  260

Git1p  253   KR---------------------------NIPYFLALKFYWKRLLGTCGTWFMYDFVTFP  285
              KR                          +I        Y + +L          F
hGlut2 261   KRLRGYDDVTKDINEMRKEREEASSEQKVSIIQLFTNSSYRQPILVALMLHVAQQF----  316

Git1p  286   NGIFSSTIISSVIKDQNDLVKVAEWNLLLGVLAVLGVPIGAYLSDRIGRKYTLMFGFSGY  345
              +GI      S+ I     + K     + +G + ++    +  +L ++ GR+    + G SG
hGlut2 317   SGINGIFYYSTSIFQTAGISKPVYATIGVGAVNMVFTAVSVFLVEKAGRRSLFLIGMSGM  376

Git1p  346   IIFGLIIGCAYDQLKKITPL---FIIFYAFMNMLGNAGPGDMLGVISSEASATAVRGVFY  402
               +  + +      L K + +    +I           GPG +   + +E  +   R
hGlut2 377   FVCAIFMSVGLVLLNKFSWMSYVSMIAIFLFVSFFEIGPGPIPWFMVAEFFSQGPRPAAL  436

Git1p  403   GLSAVTGKIGSVVGVECFQPIRDNLGARWTFIIAAICGLIGIIITYFFVPHSLESDLMKQ  462
               ++A +    + +   CFQ I D  G    F+ A +  L   + T+F VP +       +
hGlut2 437   AIAAFSNWTCNFIVALCFQYIADFCGPYVFFLFAGVL-LAFTLFTFFKVPETKGKSFEEI  495

Git1p  463   DVEFHNYLVSNGW  475
                EF     S
hGlut2 496   AAEFQKKSGSAHR  508
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Fig. 1. Sequence alignment of Gitlp and hGlut2. PSI-BLAST alignment of amino acids 84–475 of Gitlp (accession no. P25346) and 92–508 of hGlut2 (accession no. PI1168). As indicated, the alignment shows 81/433 (18%) common identities, 145/433 (33%) positives (+; semi-conserved residues that have a positive score from the BLOSUM-62 matrix [35]), and 57/433 (13%) gaps. The highly conserved residues for the Glut family members are highlighted in yellow, and the MFS-specific sequence motifs in blue [26]. The predicted membrane spanning helices (from helices 2 to 12) are underlined.

unofluorescence analysis indicated a specific plasma-membrane staining for the hGlut2 transporter in *GLUT2*-transfected cells (Fig. 2B), which was absent in mock-transfected HeLa cells.

Since Glut2 has been reported to specifically transport glucosamine [26], we verified the functionality of over-expressed hGlut2 by evaluating glucosamine (500 μM) uptake. After 5 min of incubation at 37 °C, the rate of glucosamine uptake was over twofold higher in *GLUT2*-transfected cells, as compared to mock-transfected cells (1.63 and 0.74 nmoles/min/$10^6$ cells, respectively). Altogether these data indicate that the over-expressed hGlut2 transporter correctly localizes at the plasma membrane and that it functions in the glucosamine uptake assays.

### 3.3. Characterization of GroPIns transport

GroPIns transport was evaluated by determining the time-course and concentration dependence of GroPIns uptake in *GLUT2*-transfected HeLa cells (see Section 2). As shown in Fig. 2C, the GroPIns uptake (100 μM, 37 °C) was rapid (within 1 min reaching ~75% of that seen at 15 min) and significantly higher than in the mock-transfected HeLa cells.

Similar time-course assays were performed with HEK293 cells, which endogenously express the hGlut2 transporter (Fig. 2A, right panel). The extent of GroPIns uptake observed at 37 °C is reported in Fig. 2D, and it is compatible with the proposal that endogenously expressed Glut2 can function as a GroPIns transporter.

To determine whether a fraction of GroPIns was bound to membranes (rather than internalized), we used two different approaches. In the first, total GroPIns "uptake" measured in intact cells was compared with the [$^3$H] GroPIns associated with cell lysates. In Fig. 3A, the difference in total cell-associated [$^3$H] GroPIns between the intact and lysed cells (40% for mock-transfected cells and 370% for *GLUT2*-transfected cells) represents the GroPIns uptake, with the difference between the uptake in mock- and *GLUT2*-transfected intact cells indicating

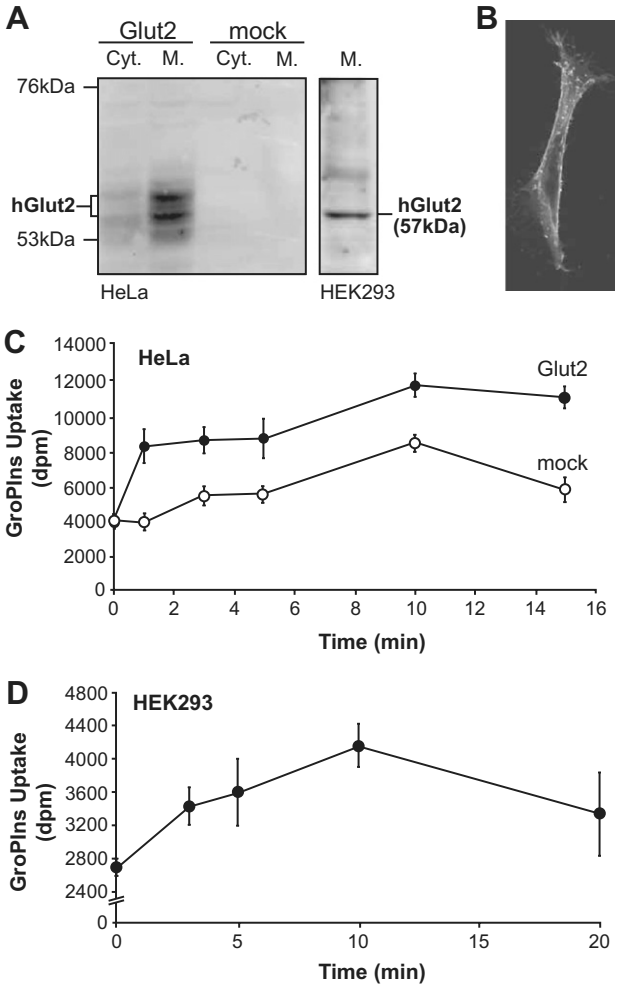


Fig. 2. Characterization of GroPIns transport. (A) Western blotting with anti-hGlut2 antiserum shows specific bands in the membrane preparation (M.) from *GLUT2*-transfected HeLa cells (left panel); and in a plasma membrane preparation (M.) from HEK293 cells (right panel). Cyt., cytosol. (B) Immunofluorescence staining of *GLUT2*-transfected HeLa cells with anti-hGlut2 antiserum, showing specific staining at the plasma-membrane. (C) Time courses of GroPIns (100 μM, 37 °C) uptake in mock- and *GLUT2*-transfected HeLa cells, expressed as dpm. Data are means ± S.E. from four independent experiments, each performed in quadruplicate; a significant increase in uptake is seen, $P < 0.02$. (D) Time course of GroPIns (250 μM, 37 °C) uptake in HEK293 cells, expressed as dpm. Data are means ± S.E. from four independent experiments, each performed in quadruplicate.

GroPIns entry through the over-expressed transporter (340%). This was also repeated in HEK293 cells, where the increase in the GroPIns uptake was ~474%, corresponding to 0.48 pmoles/min/$10^6$ cells (Fig. 3B).

The second approach involved the use of permeabilized cells, where there is no significant GroPIns uptake and the GroPIns associated with the cells is bound to either membranes or other cellular structures. In this case, GroPIns uptake (100 μM) with time was determined while comparing intact cells and cells permeabilized with tetanolysin (see Section 2; Fig. 3C). As expected, the intact cells over-expressing the transporter showed a clear increase in incorporated radioactivity, indicating uptake of GroPIns under these conditions. Moreover, the permeabilized, *GLUT2*-transfected cells showed a slightly higher binding to the membrane compared to the permeabilized mock-transfected cells, suggesting that over-expression of the transporter per se can induce an increase in GroPIns binding to cellular membranes (Fig. 3C).

Similar experiments were performed in intact and tetanolysin-permeabilized HEK293 cells. Again, a significant difference between intact and permeabilized HEK293 cells was seen with time, demonstrating further that in cells endogenously expressing the hGlut2 transporter, it is indeed possible to measure specific GroPIns uptake (Fig. 3D).

The apparent affinity of hGlut2 for GroPIns was measured in uptake experiments using 0.1–100 μM GroPIns, with mock- and *GLUT2*-transfected HeLa cells (Fig. 4). The GroPIns uptake was concentration dependent, and the $K_m$ of 80 μM was well within the intracellular concentration range of GroPIns (Fig. 4) [6].

Altogether, these data indicate that the hGlut2 transporter can mediate the uptake of GroPIns in different cell types.

### *3.4. Glucosamine competition, phloretin inhibition and RNAi*

To further demonstrate that the incorporation of GroPIns occurs through the Glut2 transporter, the effects of potential competitors, blockers and siRNAs were evaluated. First, both mock- and *GLUT2*-transfected cells were incubated with 10 μM GroPIns in the absence and presence of glucosamine, as detailed in Fig. 5A. Under these conditions, GroPIns uptake was reduced by ~35%.

The specificity of GroPIns uptake by the hGlut2 transporter was then evaluated in competition assays using small molecules such as inositol and glucose. However, GroPIns uptake at 37 °C is very rapid (Fig. 5A) making the evaluation of competition curves difficult. The competition assays for glucose and inositol were therefore performed at 25 °C, which resulted in a lower rate of GroPIns uptake. Under these conditions, inositol (10 mM) and glucose (100 μM, 1 mM) were not seen to compete with the uptake of GroPIns (50 μM) after a 10-min incubation of *GLUT2*-transfected HeLa cells; in contrast, 10 mM glucose induced an ~20% reduction and 40 mM a complete blockage in GroPIns uptake under these conditions.

Moreover, phloretin (300 μM), a blocker of the Glut2 glucose transporter that did not have any non-specific effect per se in modifying GroPIns binding to cell lysates, inhibited GroPIns uptake in both *GLUT2*-transfected cells and HEK293 cells by ~50% (Fig. 5B and C).

Finally, siRNAs specific for hGlut2 were transfected into HEK293 cells; these decreased the Glut2 levels by ~30–40% (compared with siCONTROL non-targeting-duplex-treated cells), without affecting the levels of actin, which was monitored as a control (Fig. 5E). This loss of hGlut2 caused an ~40% decrease in GroPIns uptake (Fig. 5D), confirming that this transporter can indeed mediate GroPIns uptake in cells.

## 4. Discussion

In this study, we demonstrate that in mammalian cells the human permease Glut2, a member of the MFS of transporters, can function as a transporter for the phosphoinositide derivative GroPIns. This mechanism of membrane permeation is

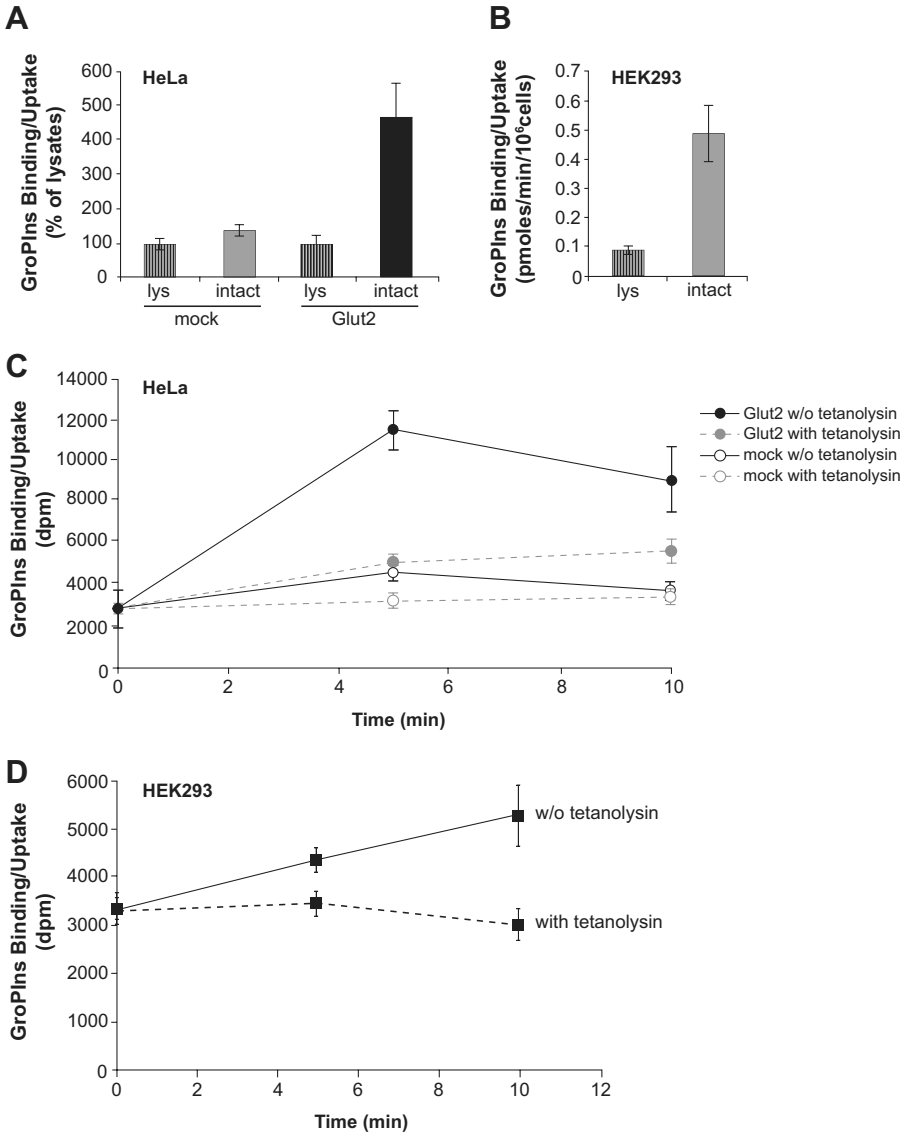


Fig. 3. GroPIns binding and uptake. (A) GroPIns binding/uptake (100 μM, 15 min, 37 °C) in intact mock- and *GLUT2*-transfected HeLa cells and in the parallel HeLa cell lysates (lys), expressed as percentages of [$^3$H] GroPIns associated with the cell lysates (see Section 2). The value of GroPIns binding to mock-HeLa cell lysates was 10 ± 1.8 pmoles/min/$10^6$ cells. Data are means ± S.E. from four independent experiments, each performed in quadruplicate. The value of *GLUT2*-transfected intact HeLa cells is significantly higher than that of mock-transfected, $P < 0.02$. (B) As for A, with HEK293 cells, expressed as pmoles/min/$10^6$ cells. Data are means ± S.E. from three independent experiments, each performed in quadruplicate. (C) GroPIns binding/uptake (100 μM GroPIns, 37 °C) in intact and tetanolysin-permeabilized mock- and *GLUT2*-transfected HeLa cells (as indicated), expressed as dpm. Data are means ± S.E. from three independent experiments, each performed in quadruplicate. (D) As for C, with HEK293 cells. Data are means ± S.E. from three independent experiments, each performed in quadruplicate.

relevant for both the metabolism of cellular GroPIns and for its pharmacological action when added exogenously to cells.

The only known, specific GroPIns transporter cloned and biochemically characterized so far is yeast Gitlp [16,17], which belongs to the Phosphate:$H^+$ symporter (PHS) subfamily of the MFS transporters, which includes members from yeast, fungi and plants, but not from bacteria, animals and other eukaryotes [27]. Based on both phylogeny and function, the classification of the MFS proteins gives no indications toward the identification of the human Gitlp ortholog, which would also indicate that this ortholog may have a low homology with the yeast protein [27]. On the other hand, the phylogenetic tree shows that the PHS and Sugar Porter (SP) families are related, and the conserved domains of the Gitlp sequence have a high score relative to the sugar-transporter domain characteristics of the members of the SP family. It is therefore not surprising that the human protein that shows the highest homology with the Gitlp through the PSI-BLAST search is Glut2, a member of the SP family (Fig. 1).

The hGlut2 protein is characterised by 12 membrane-spanning (TMS) helices, comprising seven conserved glycine residues (crucial for the structures of helices 1, 2, 4, 5, 7, 8, and 10), the QLS motif in helix 7 and the STSIF motif in loop 7 (which participate in substrate recognition), a five-residue motif (RXGRR) located between TMS2 and TMS3, and a similar motif between TMS8 and TMS9 (which is involved

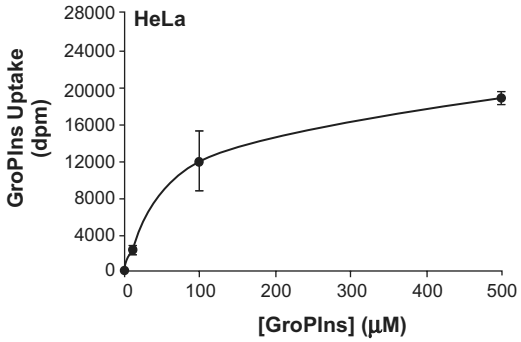


Fig. 4. Concentration dependence of GroPIns uptake in HeLa cells. GroPIns uptake (2 min, 37 °C) in *GLUT2*-transfected HeLa cells, after subtracting the uptake in mock-transfected cells, measured in the presence of increasing concentrations of GroPIns. Data are means ± S.E. of three experiments, each performed in quadruplicate.

in interactions with negative charges of membrane-lipid head groups) [24,27,28]. With the exception of the QFS motif, these are also present in the Gitlp sequence (Fig. 1).

Based on sequence homology, Glut2 was originally identified as a glucose-like transporter [29]. Glut2 function has been in part explained with the model of intestinal sugar transport, where dietary glucose is transported "uphill" by active transporters (such as the $Na^+$/glucose co-transporter, SGLT1) and then exits across the basolateral membrane of enterocytes through the facilitative Glut2 transporter [30]. Similar mechanisms have been proposed for glucose transport in hepatic and pancreatic beta cells, even if Glut2-knock-out mice show evidence of passive hepatic membrane glucose transport that is Glut2-independent [31].

More recently, however, Glut2 has been shown to have a much higher apparent affinity for glucosamine (a naturally occurring sugar that is necessary for the synthesis of proteoglycans) than for glucose ($K_m = 0.8 \pm 0.1$ mM versus ~17–20 mM, respectively) [26]. Despite this, the affinity of Glut2 for glucosamine remains in a relatively non-physiological [26,32], low millimolar range, suggesting that the endogenous ligand(s) of this transporter remains to be defined.

This is further supported by the data presented in this study, where we have defined a 10-fold higher apparent affinity of Glut2 for GroPIns ($K_m = 80$ μM), with respect to the data reported in oocytes for glucosamine [26].

According to our observations, Glut2 transports in a competitive manner both positively (glucosamine) and negatively (GroPIns) charged compounds, though the mechanism involved remains to be clarified. A similar competition by differently charged compounds has been reported in the case of Gitlp in yeast, where the transport of GroPIns is partially competed by zwitterionic compounds, such as glycerophosphocholine and glycerophosphoethanolamine [16].

This characterization of Glut2 as a mammalian ortholog of Gitlp is also compatible with the cellular concentrations of GroPIns [6], and the lack of competition by physiological concentrations of glucose or glucosamine clearly point to the specificity of Glut2-mediated GroPIns transport. In line with these results, the downregulation of Glut2 expression by siRNAs also significantly decreased GroPIns uptake (Fig. 5D).

In addition, the features of Glut2-mediated GroPIns cellular uptake are in line with those of the glucose uptake that is

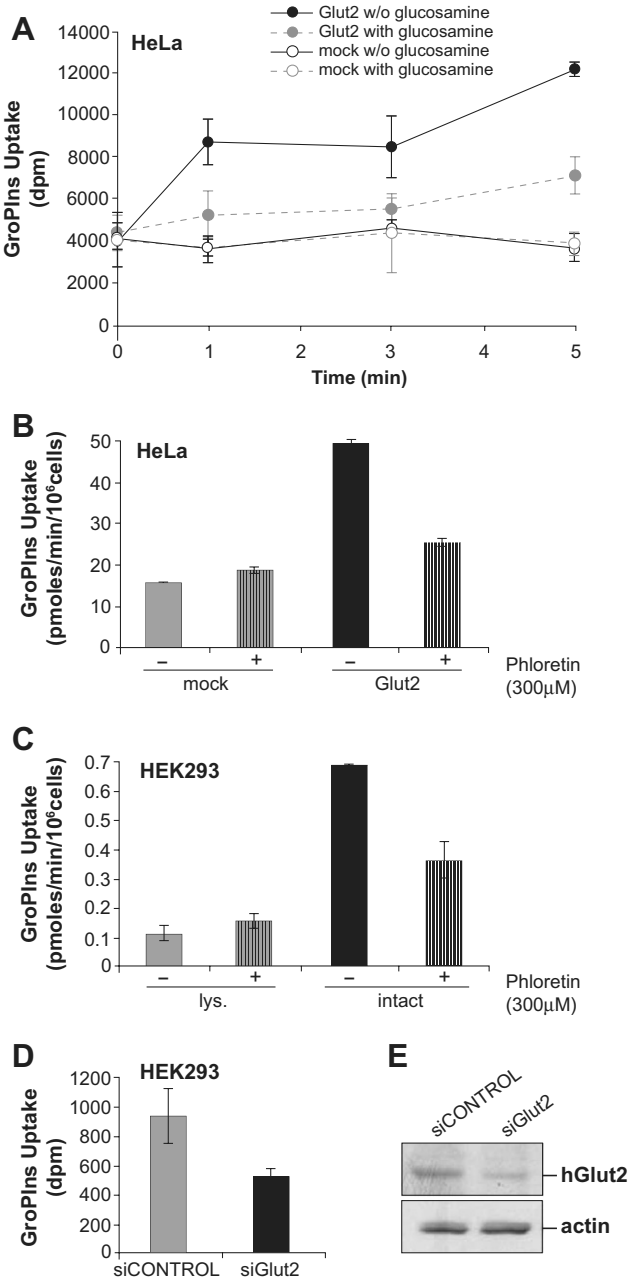


Fig. 5. Glucosamine competition, phloretin inhibition of GroPIns uptake. (A) Time courses of GroPIns uptake (10 μM, 37 °C) in intact mock- and *GLUT2*-transfected HeLa cells in the absence and presence of 100 mM glucosamine (as indicated), expressed as dpm. Data are means ± S.E. from three independent experiments, each performed in quadruplicate. (B) GroPIns uptake (100 μM, 10 min, 37 °C) in mock- and *GLUT2*-transfected HeLa cells in the absence and in presence of 300 μM phloretin (as indicated), expressed as pmoles/min/$10^6$ cells. Phloretin significantly inhibits GroPIns uptake in *GLUT2*-transfected HeLa cells, $P < 0.01$. (C) As for B, with HEK293 cells. Phloretin significantly inhibits GroPIns uptake in intact HEK293 cells, $P < 0.02$. Data are means ± S.E. from three independent experiments, each performed in quadruplicate. (D) GroPIns (100 μM, 2 min, 25 °C) uptake in HEK293 cells treated with siCONTROL duplexes (siCONTROL) and with Glut2-directed siRNAs (siGlut2), expressed as dpm. Data are means ± S.E. from four independent experiments, each performed in quadruplicate, and show a significant decrease in uptake, $P < 0.05$. (E) Western blotting with anti-hGlut2 antiserum (upper panel) and anti-actin antibody (lower panel) shows a specific decrease (up to 40% in the different experiments) in the Glut2 band in siGlut2 cell lysates.

mediated by Glutl. Thus, this uptake is rapid and shows a time course that is similar to that reported for 3-*O*-methyl-D-glucose uptake into MIN6 cells (mouse insulin-secreting clonal β-cells, expressing the Glut2 and Glutl glucose transporters), which has been shown to be rapid and to reach 80% equilibrium within 1 min [33].

Of note, the association of radiolabeled GroPIns with intact cells could derive not only from the uptake of this compound but also in part from its binding to membranes. This possibility was analysed, and the net transport was resolved based on a clear increase in GroPIns uptake in intact cells versus permeabilized cells, and also on the pronounced increase of GroPIns uptake seen upon Glut2 over-expression in intact cells (Fig. 4). In addition, this increased transport was reduced by the inhibitor phloretin and, more important, by the downregulation of the Glut2 expression using siRNAs, further supporting the specificity of Glut2 for GroPIns uptake.

Altogether, these data are in line with the molecular identification of Glut2 as one of the proteins involved in GroPIns transport in mammalian cells. It cannot be concluded, however, that Glut2 is the only GroPIns transporter in mammals, since Glut2 is not ubiquitously expressed, but is instead predominantly present in liver, kidney and small intestine [29]. This indicates that either phosphoinositide/GroPIns metabolism is prevalent in these specific tissues, or that, most probably, other proteins have a transporter function in tissues where Glut2 is not expressed. That this is indeed the case is shown by the levels of GroPIns uptake revealed in cells that do not expressed Glut2, such as, for example, HeLa cells (cervix adenocarcinoma cells) (Fig. 2C) and Swiss 3T3 fibroblasts [18]. In these cases, this uptake can reasonably be assigned to a Glut2-independent mechanism. Instead, the parallel decrease in Glut2 expression and GroPIns uptake observed in kidney cells (HEK293, Fig. 5D), strengthen the conclusion that when endogenously expressed, Glut2 is the specific GroPIns transporter.

Previous studies have discussed the relevance of transmembrane GroPIns permeability for fully defining the GroPIns metabolic pathways and for exploiting its pharmacological potential. For the former, GroPIns has been detected in both the intracellular and extracellular spaces [5,19], and ecto-enzymes (e.g. glycerophosphodiesterase 1) that can specifically metabolize GroPIns to glycerol and inositol monophosphate have indeed been characterized [20].

As already mentioned, GroPIns is biologically active when administered exogenously to cells, pointing to the potential pharmacological exploitation of this compound and its derivatives. Indeed, when exposed to micromolar concentrations of GroPIns, melanoma and breast carcinoma cells have a significantly reduced ability to invade the extracellular matrix [3]. This process requires the modulation of several cell functions, including cell attachment and motility, and the interaction, digestion and penetration of the extracellular matrix [34]. Although not yet fully elucidated, the regulation of these processes involves activation of different signalling pathways and of the actin machinery, indicating that the action of GroPIns occurs after it has permeated into the cell. To date, GroPIns has been shown to activate the small GTPase Rac in fibroblasts (but not to affect actin organization [2]), and its mechanism of action in tumor cells remains under investigation.

Based on these findings, GroPIns, and more in general, the glycerophosphoinositols, have the desired characteristics (small, naturally occurring, non-toxic, and water-soluble molecules) for their exploitation in the development and application of potential antiproliferative therapies. Our present characterization of the permeation mechanism responsible for the transport of GroPIns across the cell membrane clearly further promotes their therapeutical potential and interest.

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