FEBS Letters 580 (2006) 6789–6796

Molecular characterization of a glycerophosphoinositol transporter in mammalian cells

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Received 2 August 2006; revised 4 November 2006; accepted 13 November 2006

Available online 27 November 2006

Edited by Felix Wieland

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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0014-5793/$32.00
doi:10.1016/j.febslet.2006.11.039

(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-