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Phosphorylation of glycosyl-phosphatidylinositol by phosphatidylinositol 3-kinase changes its properties as a substrate for phospholipases

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Abstract Phosphatidylinositol 3-kinases (PI3K) phosphorylate the 3-position of the inositol ring of phosphatidylinositol-4,5-bisphosphate to produce phosphatidylinositol-3,4,5-trisphosphate. It is not clear whether PI3K can phosphorylate the inositol group in other biomolecules. We sought to determine whether PI3K was able to use glycosyl-phosphatidylinositol (GPI) as a substrate. This phospholipid may exist either in free form (GPI^{free}) or forming a lipid anchor (GPI^{anchor}) for the attachment of extracellular proteins to the plasma membrane. We demonstrate the specific PI3K-mediated phosphorylation of the inositol 3-hydroxyl group within both types of GPI by incubating this phospholipid with immunoprecipitated PI3K. The phosphorylated product behaves in HPLC as a derivative of a PI3K lipid product. To our knowledge, this is the first demonstration that PI3K uses lipid substrates other than phosphoinositides. Further, we show that this has potential functional consequences. When GPI^{free} is phosphorylated, it becomes a poorer substrate for GPI-specific phospholipase D, but a better substrate for phosphatidylinositol-specific phospholipase C. These phosphorylation events may constitute the basis of a previously undescribed signal transduction mechanism.

Keywords: Phosphorylation; Phosphatidylinositol 3-kinase; Glycosyl-phosphatidylinositol

1. Introduction

Phosphoinositides are now widely recognized as playing key roles in both signal transduction pathways and in the control of multiple intracellular activities [1]. Two signal transduction pathways involving inositol-containing phospholipids have been characterized to date. The best characterized is the one involving agonist-stimulated generation of the second messengers diacylglycerol and inositol-1,4,5-triphosphate, respectively causing activation of protein kinase C and liberation of calcium ions from intracellular stores [2–4]. In addition, a new family of 3-phosphorylated phosphoinositides has been identified in cells that are derived through the action of PI3K. There are three structurally distinct classes of PI3K [5]. The Class I PI3K subfamily is activated as a consequence of the stimulation of receptor tyrosine kinases/G-protein coupled receptors causing the initial production of phosphatidylinositol-3,4,5-trisphosphate (PtdIns3,4,5P_3) from its preferred substrate PtdIns4,5P_2 [6]. The Class II PI3K subfamily preferentially uses phosphatidylinositol-4-monophosphate (PtdIns4P) and phosphatidylinositol (PtdIns) as substrates, generating phosphatidylinositol-3,4-bisphosphate (PtdIns3,4P_2) and phosphatidylinositol 3-monophosphate (PtdIns3P) [7,8]. Finally, Class III PI3K is only able to generate PtdIns3P [9]. Our understanding of the consequences of PI3-K activation derives mainly from studies on the Class I family. Our current view is that activation of PI3K leads to a plethora of cell responses, including cell proliferation, cell differentiation, cell survival and cell motility [10,11].

Glycosyl-phosphatidylinositol (GPI) lipids display a dual role, as anchors for membrane-associated proteins and as protein-free precursors for the generation of biologically active IPG molecules [12–16]. GPI^{free} consists of PtdIns bound to a 2-amino-2-deoxy-α-D-glucopyranose through a 1-D–4-O-bond. The latter is subsequently linked to a phosphorylated glycan. GPI^{free} is strongly implicated in mediating numerous actions of hormones, resulting in metabolic changes within the cell, and of growth factors and cytokines, leading to proliferation and differentiation [12–14]. In response to insulin, the classical PtdIns cycle is not activated. In contrast, both the GPI^{free} and the PI3K pathways are activated. Failure to do so results in the loss of the metabolic actions of insulin [13,17]. Although the PI3K family of enzymes is known to phosphorylate PtdIns, PtdIns4P, and PtdIns4,5P_2, there are no reports in the literature concerning enzymatic phosphorylation of the inositol moiety of GPI^{free} nor of GPI^{anchor}. In the present paper, we describe the specific in vitro 3-phosphorylation of the inositol ring within GPI^{free} (isolated from rat liver) and also of GPI^{anchor} [isolated from bovine erythrocyte acetylcholine esterase (AChE)] by mammalian Class I PI3K. As a

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consequence, phosphorylated GPI^{free} (GPI^{free}–P) may exert some control on the metabolic actions of insulin as GPI^{free}–P displays a different susceptibility to hydrolysis by specific phospholipases. This would constitute a third, novel signal transduction pathway involving phosphoinositides.

2. Materials and methods

2.1. Materials

[γ^{32}P]ATP (specific activity 3000 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Amersham, UK). Foetal calf serum, cell culture media and supplements were supplied by Gibco (Paisley, UK). Silica gel thin layer chromatography (TLC) plates (60 Å, LK6D) were from Whatman (Maidstone, UK). The anti-p85 antibody was from Upstate Biotechnology Inc (Waltham, MA, USA). Phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEth), phosphatidic acid (PtdOH), PtdIns4P, PtdIns, wortmanin, LY294002, and protein-A Sepharose were from Sigma (Poole, UK). Authentic radiolabelled standards for strong ion exchange-high performance liquid chromatography (SAX-HPLC) were prepared as previously described [18]. phosphatidylinositol-specific phospholipase C (PI-PLC) (*Bacillus thuringiensis*) was from Molecular Probes Inc. (Eugene, OR, USA) and bovine brain GPI-specific phospholipase (GPI-PLD) was purified as reported [19]. Salts, organic solvents and other reagents were of analytical grade and were supplied by Merck (Darmstadt, Germany).

2.2. Purification of GPI^{free} and GPI^{anchor}

GPI^{free} was purified as detailed [20]. Briefly, total lipids isolated by $CHCl_3$/MeOH/HCl extraction of 30 rat livers were applied to the origin of heat-activated TLC plates. The plates were then developed twice in the acidic solvent system consisting of $CHCl_3$/CH_3COCH_3/MeOH/CH_3COOH/H_2O (10/4/2/2/1, v/v). The material remaining at the origin (from −0.5 cm below to +1 cm above) was eluted from the silica by washing three times with methanol. The eluted material was dried under a gentle stream of nitrogen gas before it was applied to a further set of TLC plates for development in the basic solvent system consisting of $CHCl_3$/MeOH/16 M NH_3/H_2O (45/45/3.5/10, v/v). GPI^{free} was located between the authentic standards PtdOH and PtdCho, which had been spotted onto the plates. Confirmation that GPI^{free} was indeed the lipid migrating at the stated position was achieved by noting its reactions with iodine vapour, 1,6-diphenylhexatriene (both general lipid stains), the orcinol reagent (specific for sugar residues), ninhydrin (amino group detector) and the molybdenum blue reagent (for phosphate). GPI^{free} that had not been exposed to the detecting reagents was removed from the silica using methanol as described above. The yield of GPI^{free} was calculated from its free amino group content and its total number of phosphate groups measured by previously published methods [21]. It has been proposed that the stoichiometry of the number of phosphate groups to free amino groups is 3:1 [21]. To confirm the absence of polyunsaturated fatty acids (to rule out the possibility of contamination of the GPI^{free} preparation by phosphoinositides), gas chromatography-mass spectrometry (GC/MS) was used as previously described [22]. Acetylcholinesterase from bovine erythrocytes was purified as described [23]. To the pure enzyme (1.1 mg in 2.5 ml of 20 mM Tris–HCl, pH 7.4, containing 5 mM $CaCl_2$ and 0.05% Triton-X 100), 3.2 mg pronase (F. Hoffmann-La Roche Ltd., Basel, Switzerland) was added and the mixture incubated for 21 h at 37 °C. The GPI^{anchor} was purified by chromatography on an octyl-Sepharose column (1.5 ml) as described [24]. In order to separate the GPI^{anchor} from the peptides that resulted from the pronase digestion, the column was first washed with 10 ml of 5% propan-1-ol in 0.1 M ammonium acetate buffer, pH 7.0, followed by 35 ml of 20% propan-1-ol in the same buffer. The GPI^{anchor} was then eluted with 40% propan-1-ol in ammonium acetate buffer. Anchor containing fractions were pooled and concentrated by evaporation under nitrogen.

2.3. Class I PI3-K immunoprecipitation and phosphorylation of substrates

The IL-2-dependent murine T-lymphocyte cell line CTLL-2 was maintained in suspension culture as previously described [18]. 1×10^8 cells obtained from an exponentially growing culture were washed twice in PBS before their lysis in ice-cold lysis buffer (150 mM NaCl, 50 mM NaF, 1% v/v NP-40, 1 mM EDTA, 1 mM orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride) for 15 min. After centrifugation at 4 °C for 15 min at $20\,800 \times g$, the cleared supernatant was mixed with 5 μg of anti-p85 antibody. Immunoprecipitation was allowed to proceed for 2 h at 4 °C with gentle agitation. Protein-A Sepharose (approximately 80 μl packed volume) was then added and the mixing was continued for a further one hour. The Sepharose beads were then washed twice with lysis buffer, twice with 0.5 M LiCl and finally twice with PI3K assay buffer (50 mM Tris–HCl, pH 7.4). The immunoprecipitate was divided into two portions. 90% was used for the phosphorylation of either GPI^{free} or GPI^{anchor}, whereas 10% was used to phosphorylate PtdIns. The substrates (10 nmol of GPI^{free}, 5 μg GPI^{anchor} or 1 nmol of PtdIns) were sonicated (10 min at room temperature) into 20 μl of PI3K assay buffer. The final reaction conditions contained ATP at 20 μM, $MgCl_2$ at 10 mM and 25 μCi [γ^{32}P]ATP in a final volume of 50 μl. The reaction was allowed to proceed at room temperature for 15 min before the extraction of lipids with $CHCl_3$/MeOH/HCl. The $CHCl_3$ phase was taken into a clean glass tube and dried under a gentle stream of nitrogen gas. After resuspending in chloroform, a portion of the samples was applied to a TLC plate, which was developed in a basic solvent system (see above). After development, the TLC plate was allowed to dry before staining with iodine vapour. Authentic phospholipid standards (including unlabelled GPI^{free}), which had been applied to the TLC plate alongside the samples, were visualized as brown spots. The positions of phosphorylated products were determined by autoradiography. In some experiments, human embryonic kidney fibroblasts (HEK-293 cells) were used as a source for Class II PI3K activity [25].

2.4. Nitrous acid deamination of reaction products and their analysis by SAX-HPLC

The remaining portion of the samples was dried under a gentle stream of nitrogen gas before treatment with nitrous acid, as previously described [21]. The deacylation of the radiolabelled lipids was achieved using methylamine. The deacylated radiolabelled lipids were analyzed by SAX-HPLC as described previously [18]. In some experiments, the nitrous acid deamination step was omitted in order to examine the phosphorylation state of deacylated GPI^{free}–P by SAX-HPLC.

2.5. Hydrolysis of GPI^{free} and GPI^{free}–P by phospholipases

Purified GPI^{free} or GPI^{free}–P (obtained by cold phosphorylation of rat liver-derived GPI^{free} by PI3K and its subsequent purification by TLC) were reconstituted asymmetrically into preformed liposomal membranes as described previously [26]. Briefly, large unilamellar vesicles composed of PtdEth/PtdCho (2:1 mole ratio) were prepared by the extrusion method [27], using 0.1 μm pore diameter Nuclepore filters at room temperature, as detailed previously [28]. Total lipid concentration was adjusted to 80 μM after lipid phosphate analysis. Then, the appropriate amount of GPI^{free} or GPI^{free}–P was dissolved in methanol and mixed with the liposomes in buffer, so that the GPI^{free} (or GPI^{free}–P) mole fraction in the lipid was 10%, unless otherwise stated, and the volume of the methanolic solution was 5% of the vesicle suspension. The resulting mixture was left to incubate for 15 min at room temperature. This causes GPI^{free} and GPI^{free}–P to become asymmetrically inserted into the outer monolayer of the vesicles. The resulting suspension was then used in the phospholipase assays using the enzymes previously described [22,26]. The assays were linear with respect to time and enzyme concentration.

2.6. Data presentation

Unless specified otherwise, data are presented as representative results from at least three independent experiments. Enzyme activity data are averages of two closely similar independent measurements.

3. Results and discussion

Insulin is a hormone that simultaneously activates multiple signal transduction pathways, including the generation of IPG [13,29–31] and the activation of PI3K [17]. In order to study possible crosstalk between these two apparently distinct pathways in a cell-free system, we decided to determine

(i) whether PI3K was able to use either GPI^{free} or GPI^{anchor} as substrates for a 3-phosphorylation reaction and (ii) whether GPI^{free}–P was susceptible to hydrolysis by the enzymes GPI-PLD and PI-PLC.

Class I PI3K was immunoprecipitated and incubated with either GPI^{free} (purified from rat liver) or commercially available PtdIns in the presence of [γ^{32}P]ATP. The reaction products were extracted with $CHCl_3$/MeOH before being separated by TLC. Fig. 1 shows the autoradiogram of the TLC plate. When the substrate was PtdIns, a radiolabelled spot ([^{32}P]PtdIns3P) that coincided with the unlabelled authentic PtdIns4P was seen. It must be noted that the solvent system employed in the TLC was unable to separate PtdIns3P from PtdIns4P. When GPI was presented as the substrate, a radiolabelled spot with a R_f of approximately 0.43 was observed. Its migration was very similar to that of [^{32}P]PtdIns3P and the unlabelled authentic PtdIns4P. This spot was putatively identified as [^{32}P]GPI^{free}-P.

In order to determine where the [^{32}P] was incorporated in the [^{32}P]GPI^{free}–P, it was necessary to chemically break down the structure for its analysis by HPLC. [^{32}P]GPI^{free}–P was treated with nitrous acid to break the bond between inositol and glucosamine, yielding a PtdIns moiety and free glycan. The [^{32}P] radioactivity was found within the PtdIns moiety and not with the free glycan. The last step in the analysis was to determine the position of the [^{32}P]phosphate group within the inositol ring. After deacylation, the GroPIns[^{32}P]phosphate was subjected to SAX-HPLC. The results of the SAX-HPLC analysis are shown in Fig. 2. In panel A, the authentic deacylated products of [^{32}P]PtdIns3P and [^{3}H]PtdIns4P ([^{32}P]GroPIns3P and [^{3}H]GroPIns4P, respectively) are clearly separated from one another, with retention times of 39.5–40 min and 43.5–44 min, respectively. In order to determine if acid-catalyzed migration of the [^{32}P]phosphate group around the inositol ring occurred during nitrous acid treatment of [^{32}P]GPI^{free}–P (for example, from position 3 to position 4), authentic [^{32}P]PtdIns3P was subjected to nitrous acid deamination in parallel to that of [^{32}P]GPI^{free}–P. Panel B clearly shows that when [^{32}P]PtdIns3P was taken through the whole procedure, over 90% of the radioactivity was present in the peak corresponding to [^{32}P]GroPIns3P, thereby ruling out this cause for concern. When the [^{32}P]GPI^{free}–P-derived GroPIns[^{32}P]phosphate was subjected to SAX-HPLC analysis two peaks were observed. The former, eluting at 40 min, contained approximately 80% of the total radioactivity in the two peaks and coincided with the elution of the authentic [^{32}P]GroPIns3P standard. When the phosphorylation of GPI^{free} was performed in the presence of the PI3K inhibitors, wortmannin (100 nM) and LY294002 (10 μM), the formation of GPI^{free}–P was significantly inhibited (by 73% and 84%, respectively, data not shown). GPI^{free} was purified using multiple TLC steps,

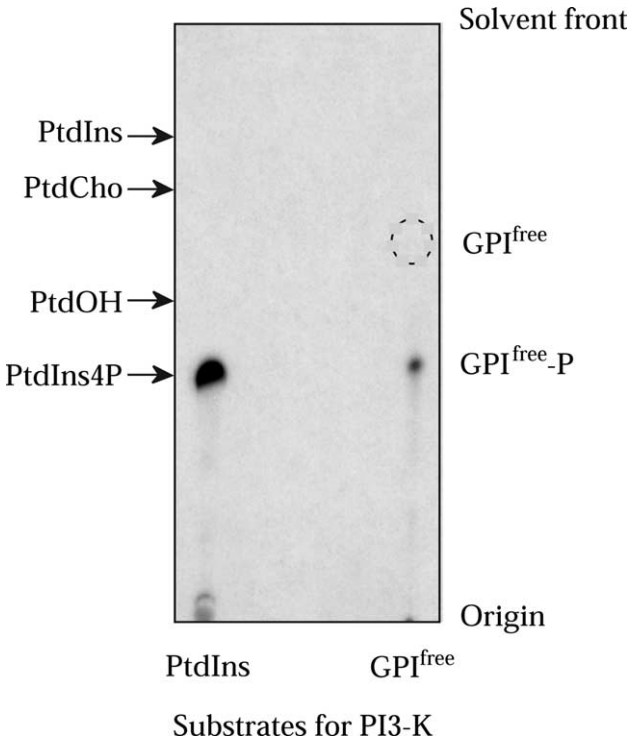

Fig. 1. TLC analysis of the in vitro phosphorylation of GPI^{free} and PtdIns by PI3K. GPI^{free} and PtdIns were used as substrates for in vitro PI3K phosphorylation. A portion of the radiolabelled products was applied to a TLC plate followed by its development in a basic solvent system. [^{32}P]GPI^{free}–P migrated with an R_f of approximately 0.43, coinciding with that of [^{32}P]PtdIns3P. The migration positions of the authentic phospholipid standards are indicated. The broken circle shows where non-radiolabelled GPI^{free} migrated (revealed by iodine staining).

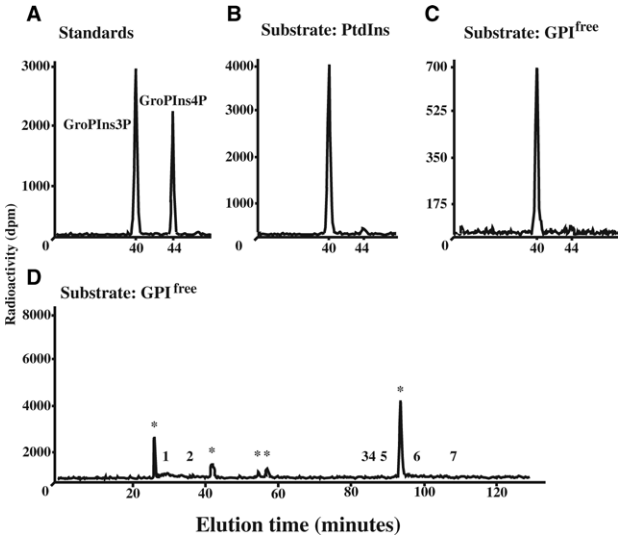

Fig. 2. SAX-HPLC analysis of the in vitro phosphorylation of GPI^{free} and PtdIns by PI3K. GPI^{free} and PtdIns were used as substrates for in vitro PI3K phosphorylation. The radiolabelled products were then treated with nitrous acid before deacylation. The deacylated lipids were analyzed by SAX-HPLC. Panel A indicates the SAX-HPLC profile of GroPIns3P and GroPIns4P derived from authentic [^{32}P]PtdIns3P and [^{3}H]PtdIns4P. Panel B indicates the SAX-HPLC profile of PtdIns, which had been previously phosphorylated by PI3K, treated with nitrous acid and deacylated. Panel C indicates the SAX-HPLC profile of GPI^{free}, which had been previously phosphorylated by PI3-K, treated with nitrous acid and deacylated. Panel D shows the SAX-HPLC profile of the deacylated reaction products of GPI^{free}, which had been previously phosphorylated by PI3K. The asterisks indicate peaks that may correspond to a family of differentially 3-phosphorylated GroPIns-glycan species. The numbers that appear within the SAX-HPLC profile in panel D refer to the position of authentic standards. 1, Gro3P; 2, inorganic phosphate 3, glycerophosphoinositol-3,5-bisphosphate; 4, glycerophosphoinositol-3,4-bisphosphate; 5, glycerophosphoinositol-4,5-bisphosphate; 6, ATP; 7, glycerophosphoinositol-3,4,5-trisphosphate.

thereby eliminating possible contamination with PtdIns which could have led to the generation of [^{32}P]PtdIns3P. Furthermore, the absence of polyunsaturated fatty acids in the GPI^{free} preparation (which would have been indicative of the presence of contaminating PtdIns) was confirmed by GC/MS ([22,32] and data not shown). To further confirm that the GPI^{free} preparation was not contaminated with PtdIns, Class I PI3K was immunoprecipitated followed by its incubation with GPI^{free} in the presence of [γ^{32}P]ATP. The reaction products were deacylated without prior nitrous acid deamination and subjected to analysis by SAX-HPLC. Fig. 2 panel D indicates the SAX-HPLC profile. No peak corresponding to GroPtdIns3P was found to be present, thereby confirming that the GPI^{free} preparation was not contaminated with PtdIns. Panel D also reveals that several peaks of radioactivity were present in the SAX-HPLC profile (marked with an asterisk) whose absolute identification remains to be determined. These peaks most likely correspond to a series of differentially 3-phosphorylated GroPIns-glycan species. This observation of multiply phosphorylated GPI^{free} species has been shown previously and is reflected in the chromatographic characteristics of both IPG [33] and GPI^{free} [21,34].

After determining that PI3K was able to phosphorylate rat liver-derived GPI^{free}, we decided to ascertain whether GPI^{anchor} was also a substrate for PI3K. GPI^{anchor} isolated from bovine erythrocyte AChE (GPI$^{AChE\text{-}anchor}$) was incubated with immunoprecipitated PI3K and [γ^{32}P]ATP. After performing a lipid extraction, a [^{32}P]labelled product migrated on a TLC plate with an R_f of approximately 0.53 (Fig. 3, panel A). When an active recombinant Class I (p85α/p110α) PI3K was used instead of PI3K immunoprecipitated from mammalian cells, a [^{32}P]labelled product with similar chromatographic properties was generated (data not shown). The structures of GPI^{free} and GPI^{anchor} show both similarities and differences. The latter are apparent when their glycan cores are compared [12,35]. Despite this, the R_f of the [^{32}P] labelled product [putatively identified as phosphorylated bovine erythrocyte AChE GPI^{anchor} (GPI$^{AChE\text{-}anchor}$–P)] was only slightly higher than GPI^{free}–P when GPI^{free} was used as a substrate in an identical in vitro phosphorylation reacion (Fig. 1). In order to confirm that the [^{32}P]GPI$^{AChE\text{-}anchor}$–P was indeed 3-phosphorylated, it was necessary to use the analysis method employed previously. The [^{32}P]GPI$^{AChE\text{-}anchor}$–P was treated with nitrous acid and the products were deacylated before analysis by HPLC. Fig. 3 panel B indicates that the majority of the radioactivity eluted at approximately 30 min. A peak at 40 min was also evident, which coincided with that of the [^{32}P]GroPIns3P standard. No peak at 40 min was detected after mock treatment of [^{32}P]GPI$^{AChE\text{-}anchor}$–P (data not shown). Nitrous acid deamination requires the amino group of the hexosamine residue to be free, i.e., not substituted. Our findings that only a small proportion of [^{32}P]PtdIns3P was liberated from the [^{32}P]GPI$^{AChE\text{-}anchor}$–P after treatment with nitrous acid is most likely explained by the fact that the hexosamine residue in the GPI$^{AChE\text{-}anchor}$ is naturally modified by N-methylation [36], thereby greatly hindering the nitrous acid deamination reaction. Therefore, the peak of radioactivity eluting from the HPLC at 40 min corresponds to the proportion of [^{32}P]GPI$^{AChE\text{-}anchor}$–P sensitive to nitrous acid deamination, implying that the majority is resistant due to the N-methylation. Nitrous acid deamination of GPI^{free}–P proceeds without hindrance, as the hexosamine residue has no amino group substitutions. Immunoprecipitated PI3K preparations underwent both analysis by TLC and HPLC to eliminate the possibility that [^{32}P]PtdIns3P (and hence [^{32}P]GroPIns3P) could have been generated in incubations in the absence of substrates. The major peak of radioactivity, which elutes from the HPLC column at 30 min, coincided with that of the [^{32}P]Gro3P standard. However, the generation of [^{32}P]PtdOH during the in vitro phosphorylation of GPI$^{AChE\text{-}anchor}$ was extremely unlikely as both immunoprecipitated PI3K and GPI$^{AChE\text{-}anchor}$ contained no measurable DAG, immunoprecipitated PI3K contained no diacylglycerol kinase activity and [^{32}P]GPI$^{AChE\text{-}anchor}$–P migrated on a TLC plate differently from that of PtdOH.

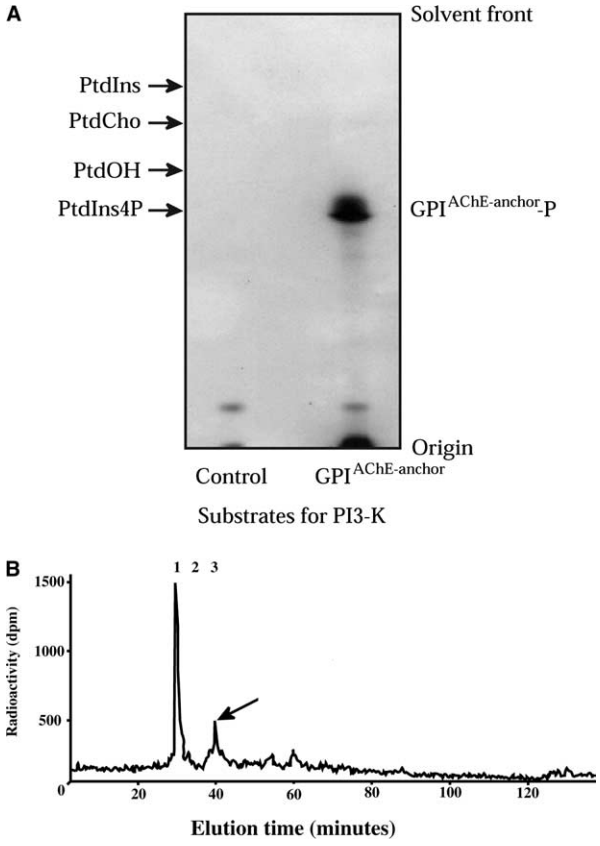

Fig. 3. TLC and SAX-HPLC analysis of the phosphorylation of GPI$^{AChE\text{-}anchor}$ by PI3K. Panel A. Buffer (control) and GPI$^{AChE\text{-}anchor}$ were used as substrates for in vitro PI3K phosphorylation. The radiolabelled product was applied to a TLC plate followed by its development in a basic solvent system. [^{32}P]GPI$^{AChE\text{-}anchor}$–P migrated with an R_f of approximately 0.53, coinciding with that of [^{32}P]PtdIns4P. The migration positions of the authentic phospholipid standards are indicated. Panel B. [^{32}P]GPI$^{AChE\text{-}anchor}$–P recovered from the TLC plate was subjected to nitrous acid deamination followed by its deacylation. The radiolabelled products were then analyzed by SAX-HPLC. The arrow indicates the minor peak identified as [^{32}P]GroPIns3P. The numbers above the SAX-HPLC profile refer to the position of authentic standards. 1, Gro3P; 2, inorganic phosphate; 3, GroPIns3P.

Together, these results demonstrate that PI3K is able to 3-phosphorylate the inositol ring of both GPI^{free} and at least one GPI^{anchor}. To the authors' knowledge, this is the first report of GPI phosphorylation by PI3K. There are many different species of GPI^{anchor}, therefore it would be of future interest to determine if PI3K is able to phosphorylate all of them or

rather a selected subset. Since both GPI^{free} and PI3K are important molecules in the control of cell proliferation, and in particular they are both significant insulin mediators [10–13,29–31], the fact that GPI^{free} can be a substrate of PI3K is undoubtedly important, and these in vitro observations will lead to in depth in vivo experiments.

The above results were obtained by using Class I PI3K immunoprecipitated from mammalian cells and an active recombinant Class I (p85α/p110α) PI3K. In vivo, the Class I enzyme is not believed to use PtdIns as a substrate [5], however, in vitro, PtdIns is preferred over other phosphoinositides. In addition to the activation of Class I PI3K, insulin is also known to activate the Class II PI3K, particularly the α isoform [21,37,38]. Class II PI3K may be associated to membrane subdomains such as caveolae, the same compartment reported to be rich in insulin-sensitive GPI^{free} [39] and where insulin receptor binding and initiation of downstream signalling pathways take place [14,40]. In order to determine if Class II PI3K was able to 3-phosphorylate GPI^{free}, we immunoprecipitated the enzyme from HEK-293 cells [25]. TLC analysis of the reaction products revealed that Class II PI3K was also able to phosphorylate GPI-^{free} (data not shown). Together, we have confirmed that both Class I and Class II PI3-K enzymes possessed the ability to phosphorylate GPI^{free} in vitro. One major question is where cellular phosphorylation of both GPI^{free} and GPI^{anchor} could occur. Our data suggest that both GPI^{free} and GPI^{anchor} phosphorylation could occur at submembrane compartments where the close association between PI3K, specific phospholipases and their substrates would be facilitated. Different in vivo and in vitro experiments support this hypothesis. Firstly, all the elements have been reported to be located and even activated into those compartments [39–46]. Secondly, GPI hydrolysis by phospholipases is modulated in vitro by the lipidic environment [22,26,47]. Thirdly, a recent report indicates the presence of at least two different pools of GPI precursors: the major one is not used for the synthesis of GPI^{anchor} and is located into triton-insoluble microdomains [48] suggesting that those caveolae-like membrane regions are very rich in GPI^{free}.

Phospholipases are crucial enzymes for the generation of lipid second messengers and the release of water-soluble phospholipid headgroups. GPI^{free} is a substrate for several phospholipases, mainly the parasite GPI-PLC and the mammalian GPI-PLD, however the less specific bacterial enzyme PI-PLC is also active [12,21,22,26]. In order to study whether phosphorylation of GPI^{free} induced any changes in its role as a substrate for the generation of IPG molecules, GPI^{free} and GPI^{free}–P were separately incorporated asymmetrically in the outer monolayer of large unilamellar vesicles [26]. The resulting liposomes were incubated with either PI-PLC or GPI-PLD. The time-course of enzyme hydrolysis of GPI^{free} and GPI^{free}–P is shown in Fig. 4. With PI-PLC (Fig. 4 panel A), GPI^{free}–P was hydrolyzed to a greater extent than GPI^{free}, although the initial rates were rather similar. GPI^{free} phosphorylation had a more marked effect when GPI-PLD was used (Fig. 4 panel B). Phosphorylation decreased both the rate and the extent of GPI^{free} hydrolysis by GPI-PLD. In addition, hydrolysis of GPI^{free}–P by this enzyme appears to exhibit a lag time, although the scarcity of substrate prevented us from carrying out more detailed studies. The observations in Fig. 5 were qualitatively similar in experiments at varying substrate concentrations: PI-PLC-mediated hydrolysis of GPI^{free}–P was greater than that for GPI^{free}. The reverse was found with

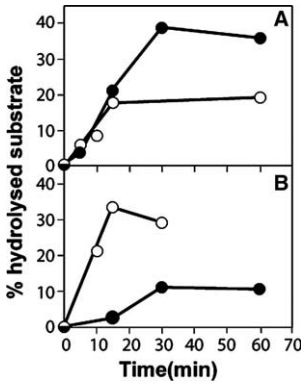

Fig. 4. Time-course of enzymatic cleavage of GPI^{free} and GPI^{free}–P. GPI^{free} (○) and GPI^{free}–P (●) were subjected to hydrolysis by PI-PLC (panel A) and GPI-PLD (panel B) at the times indicated. The initial substrate concentrations were 8 μM (panel A) and 12 μM (panel B). The enzyme concentration was in both cases 0.16 U/ml.

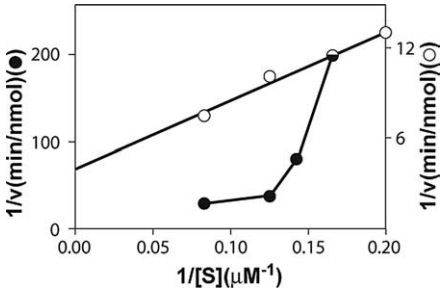

Fig. 5. Double reciprocal plot of GPI-PLD activity. The substrate was either GPI^{free} (O) or GPI^{free}–P (●). The substrate concentration range was 5–15 μM. The enzyme concentration was 0.16 U/ml. The regression line corresponding to the GPI^{free} experimental points can be extrapolated to obtain apparent kinetic parameters of K_M = 11 μM and V_{max} = 0.25 nmol/min. The experimental points corresponding to GPI^{free}–P do not follow a straight line, indicating the non-Michaelan behaviour of the enzyme in this range of substrate concentrations.

GPI-PLD. A more detailed study was performed with GPI^{free} and GPI^{free}–P using bovine brain GPI-PLD and substrate concentrations in the 5–15 μM concentration range (Fig. 5). The double-reciprocal plot shows that, when GPI^{free} was used as the substrate, GPI-PLD appears to act as a Michaelian enzyme, and the data can be adjusted to a straight line whose extrapolation provides apparent K_M and V_{max} values of 11 μM and 0.25 nmol/min, respectively. However, with GPI^{free}–P as the substrate the rates are consistently slower (note the different *Y*-axis scale) and the enzyme behaviour is no longer Michaelian, as revealed by the non-linear experimental data in the double-reciprocal plot.

The hydrolysis of 3′-phosphorylated phosphoinositides (PtdIns3P, PtdIns3,4P_2, and PtdIns3,4,5P_3) by PLCs is unlikely. To date, no such enzyme has neither been proposed nor cloned from mammalian or any other sources. In contrast, a family of mammalian PLDs specifically directed towards PtdIns3,4,5P_3 has been identified and cloned [49]. However, its real role, either attenuating the generation of PtdIns3,4,5P_3 or the generation of PtdOH or inositol-1,3,4,5-tetrakisphosphate, remains unknown. PI3K has been shown to regulate GPI hydrolysis [50] but through a different mechanism, namely tyrosine phosphorylation of PLC-γ 2, a well-established target of PI3K, in erythropoietin-stimulated cells. Our work has

focussed on the differential effects of 3′-phosphorylation of an inositol ring on phospholipase activities. At first sight, the fact that GPI^{free}–P is a better substrate for PI-PLC than GPI^{free}, yet is a poorer substrate for GPI-PLD than GPI^{free} could be considered contradictory. One explanation for this could be the requirement for a specific molecular conformation of GPI^{free} or GPI^{free}–P in the active site of the enzyme for its hydrolysis. In the case for PI-PLC, GPI^{free}–P may give a better fit, whereas for GPI-PLD the extra phosphate group may be a hindrance. Another hypothesis centres on the possibility that GPI^{free} phosphorylation by PI3K could be a negative feedback signal that decreases the efficiency of GPI^{free} hydrolysis by mammalian GPI-PLD.

At a physiological level, further studies will be required to determine to what extent GPI^{free}–P is present under resting conditions and if after agonist stimulation the ratio of GPI^{free} –P to GPI^{free} increases or decreases. One could envisage that depending on the spatial distribution of PI3K and GPI^{free}-hydrolyzing phospholipases, GPI^{free} hydrolysis in response to agonists, such as insulin, could be carefully controlled depending on its state of phosphorylation and the localization of phospholipases. In addition, insulin-stimulation of cells leads to the release of some GPI-anchored proteins via a mechanism involving an endogenous GPI-PLC [51,52]. The release of such proteins could be tightly regulated depending on the phosphorylation state of their GPI^{anchor}. As discussed above, sites at which such events may occur are the non-clathrin-coated plasma membrane invaginations termed caveolae, which have been found to contain all the substrates and enzymatic machinery required for carrying out such processes. This would represent a novel mechanism for the regulation of the release of both IPG molecules and GPI-anchored proteins in a spatial and temporal manner.

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