Intracellular cleavage of glycosylphosphatidylinositol by phospholipase D induces activation of protein kinase C α

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Many proteins are anchored to the cell membrane by glycosylphosphatidylinositol (GPI). One of the functions proposed for the GPI anchor is as a possible mediator in signal transduction through its hydrolysis. GPI-specific phospholipase D (GPI-PLD) is a secretory protein that is suggested to be involved in the release of GPI-anchored protein from the membrane. In the present study we examined how GPI-PLD is involved in signal transduction. When introduced exogenously and overexpressed in cells, GPI-PLD cleaved the GPI anchors in the early secretory pathway, possibly in the endoplasmic reticulum, resulting in an increased production of diacylglycerol. Experiments *in vitro* and *in vivo* showed that the association of protein kinase C α (PKC α) with membranes was increased markedly by expression of GPI-

PLD in cells. Furthermore, sucrose-density-gradient centrifugation and immunofluorescence microscopy demonstrated that PKC α was translocated to the endoplasmic reticulum membrane in cells expressing GPI-PLD, in contrast with its association with the plasma membrane in cells treated with PMA. We also confirmed that the phosphorylation of c-Fos as well as PKC α itself was greatly enhanced by the expression of GPI-PLD. Taken together, these results suggest that GPI-PLD is involved in intracellular cleavage of the GPI anchor, which is a new potential source of diacylglycerol production to activate PKC α .

Key words: diacylglycerol, endoplasmic reticulum, glycosylphosphatidylinositol-specific phospholipase D.

INTRODUCTION

Glycosylphosphatidylinositol (GPI) is known to anchor many proteins to the plasma membrane. In epithelial cells, all the GPI-anchored proteins are localized to the apical domain of the plasma membrane, suggesting that the GPI anchor acts as a sorting signal for these proteins to target the apical domain [1]. The GPI-anchored proteins are also enriched in caveolae [2] or membrane domains close to caveolae [3]. Caveolae are specialized microdomains that are enriched in sphingomyelin, glycosphingolipid, cholesterol and phosphoinositides [4,5] and contain GTP-binding proteins, Src-family non-receptor tyrosine kinases, protein kinase C (PKC) and caveolin [2,3,6–8]. It has been suggested that the GPI-anchored proteins are associated with the Src-family protein kinases and induce tyrosine phosphorylation mediated by tyrosine kinase [9,10].

There is also evidence that the hydrolysis of the GPI anchor is involved in signal transduction. Treatment of cells with hormones and cytokines induces the cleavage of GPI; the released inositol-phosphoglycans or the remaining diacylglycerol (DAG) act as second messengers [11–13]. In fact, protein tyrosine kinase in protozoans and PKC in macrophages are activated by inositol-glycan and DAG moieties [14]. These observations suggest the involvement of GPI-hydrolysing enzymes in the activation of these protein kinases. It is well known that the GPI-anchored proteins are easily released from the cell surface by phosphatidylinositol-specific phospholipase C (PI-PLC) purified from bacteria, although the enzyme is not specific for GPI and is not active towards mammalian GPI anchors that are acylated on *myo*-inositol. In mammals, the only purified and well-characterized enzyme responsible for GPI cleavage is GPI-specific phospholipase D (GPI-PLD), which is not affected by *myo*-inositol acylation [15]. GPI-PLD cleaves the GPI anchor and its

glycolipid precursor, but not phosphatidylethanolamine, phosphatidylcholine, or phosphatidylinositol [16]. As we and others previously reported, GPI-PLD readily hydrolyses detergent-solubilized GPI-anchored proteins but is not active towards these substrates anchored to intact membranes [17,18]. When GPI-PLD and GPI-anchored proteins are co-expressed, GPI-anchored proteins are released intracellularly from membranes by GPI-PLD, whereas those proteins expressed on the cell surface are not released by the secreted enzyme [18–20]. These observations suggest that an intracellular compartment might provide a better environment for the cleavage of GPI anchors by GPI-PLD.

In the present study we examined the effect of overexpression of GPI-PLD on cellular signalling, demonstrating that the expression of GPI-PLD causes the production of DAG, resulting in the intracellular translocation and activation of PKC α .

MATERIALS AND METHODS

Materials

[³⁵S]Methionine, [³²P]P_i, the *sn*-1,2-DAG assay reagents system and enhanced chemiluminescence (ECL[®]) kit were purchased from Amersham Corp. (Tokyo, Japan); brefeldin A (BFA) was from Wako Chemicals (Osaka, Japan); PMA was from Sigma (St. Louis, MO, U.S.A.); TransIT[®]-LT1 polyamine transfection reagent was from Mirus Corp. (Madison, WI, U.S.A.); bacterial PI-PLC was from Funakoshi (Tokyo); monoclonal antibodies against PKC α and integrin β 3 were from Transduction Laboratories (Lexington, KY, U.S.A.); rabbit polyclonal anti-(c-Fos) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.); rhodamine-conjugated goat anti-rabbit IgG was from Dako Japan (Tokyo, Japan); Cy2-conjugated donkey anti-mouse IgG was from Biological Detection System

Abbreviations used: ARF, ADP-ribosylation factor; BFA, brefeldin A; DAG, diacylglycerol; ER, endoplasmic reticulum; GPI, glycosylphosphatidylinositol; GPI-PLD, GPI-specific phospholipase D; PA, phosphatidic acid; PI-PLC, phosphatidylinositol-specific phospholipase C; PKC, protein kinase C; PLAP, placental alkaline phosphatase.

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(Pittsburgh, PA, U.S.A.). Rabbit polyclonal antibodies against human placental alkaline phosphatase (PLAP) and human GPI-PLD and GCP372/giantin were prepared as described previously [18,21,22]. Rabbit anti-calnexin antibodies were kindly provided by Dr. I. Wada (Sapporo Medical University, Sapporo, Japan) [23]. Antibodies against the C-terminal peptide (20 residues) of ADP-ribosylation factor (ARF) were raised in rabbits and used as anti-ARF antibodies.

Construction and transfection of expression plasmids

Each cDNA encoding GPI-PLD or PLAP was inserted into pSG5 expression vector (pSG5/GPI-PLD and pSG5/PLAP) [21]. The purified plasmid (6 µg) was transfected into COS-1 cells or HeLa cells by incubation at 37 °C for 6 h with TransIT[®]-LT1 polyamine transfection reagent in a serum-free medium. Cells were incubated for a further 24 h in the complete medium containing 10 % (v/v) fetal bovine serum before use.

Metabolic labelling, immunoprecipitation and SDS/PAGE

Cells (5×10^6 cells per dish) were labelled at 37 °C for 30 min with [³⁵S]methionine in 2 ml of Eagle's minimum essential medium lacking methionine, then chased in 2 ml of the complete medium. Cell lysates and culture medium were prepared and subjected to immunoprecipitation with the indicated antibodies in combination with Protein A-Sepharose [21,24]. When indicated, cells were pulse-labelled and chased for 3 h with or without 5 µg/ml BFA, subjected to phase separation with Triton X-114 [25] and the resulting detergent and aqueous phases were immunoprecipitated as above. The immunocomplexes were analysed by SDS/PAGE [7.5 % (v/v) gel] and fluorography [21]. Apparent molecular masses were determined as described previously [21].

Quantitative determination of DAG

HeLa cells (5×10^6 cells per dish) were homogenized in PBS and separated into two portions: one was used directly for lipid extraction and the other was incubated at 37 °C for 1 h with 0.5 unit of PI-PLC before extraction. Cell extraction was by a modification of the method of Bligh and Dyer [26], with 3 ml of chloroform/methanol (1:2, v/v); 1 M NaCl was added to bring the aqueous volume to 0.8 ml. After the monophasic had been mixed, 1 ml of chloroform and 1 ml of 1 M NaCl were added to break the phase. After centrifugation at 5000 g for 2 min, the lower phase was analysed with a DAG assay reagent system in accordance with the manufacturer's protocol. In brief, samples were dried with N₂ gas and suspended in the reagent mixture containing DAG kinase, followed by incubation at 25 °C for 30 min with a tracer solution containing 5 mM ATP and [γ -³²P]ATP, allowing the conversion of DAG into ³²P-labelled phosphatidic acid (PA). After being washed and dried, the samples were dissolved in 20 µl of chloroform/methanol (95:5, v/v). The samples were applied to a silica-gel 60 plate (Merck, Darmstadt, Germany) and developed with chloroform/methanol/acetic acid (65:15:5, by vol.) as solvent. The plate was dried in air and analysed by fluorography. The ³²P-labelled PA was extracted from the relevant region of the silica gel plate and the radioactivity was counted in a scintillation counter. The DAG in each sample was quantified by plotting the radioactivity against a standard curve obtained with authentic DAG.

Cell fractionation and membrane extraction

Cell fractionation and membrane extraction were performed as described previously [14]. Cells were homogenized in homogenizing buffer [0.25 M sucrose/20 mM Tris/HCl (pH 7.5)/

2 mM EGTA/2 mM EDTA/2 mM PMSF], then centrifuged at 1000 g for 10 min. The resultant postnuclear supernatant was separated by centrifugation at 105000 g for 1 h into pellet (membranes) and supernatant (cytosol) fractions. The membranes were resuspended in the same buffer containing 1 % (v/v) Triton X-100. After being incubated on ice for 10 min, the suspension was homogenized again and centrifuged at 105000 g for 30 min to obtain a supernatant (membrane extraction) and a membrane pellet.

Sucrose-density-gradient fractionation

Subcellular fractionation was performed by two established methods. In the first method [27], a postnuclear supernatant prepared from HeLa cells (5×10^7 cells) was centrifuged at 80000 g for 16 h through a continuous sucrose gradient (10.4–40 %, w/v) in 20 mM Tris/HCl (pH 7.5)/150 mM NaCl containing a mixture of protease inhibitors, with a cushion of 1 ml of 65 % (w/v) sucrose; 17 fractions of 0.9 ml were collected. In the second method [28], the postnuclear supernatant was adjusted to contain 8.5 % (w/v) sucrose, layered on sucrose gradients with 0.6 ml each of 20 %, 30 % and 38 % (w/v) sucrose and centrifuged at 100000 g for 2 h. Fractions obtained were as follows: fraction 1 from the pellet, fraction 2 from the 38 % sucrose layer, fraction 3 from the combined 20 % and 30 % sucrose layers, and fractions 4 and 5 from the 8.5 % sucrose layer.

Analysis of the membrane association of PKC α

An assay *in vitro* for the membrane association of PKC α was carried out with membranes prepared from COS-1 cells (5×10^6 per dish) that had been transfected with the indicated plasmids. The membranes prepared as described above were incubated with or without PI-PLC (0.5 unit) at 37 °C for 1 h or with 1 µM PMA at 37 °C for 20 min. The membranes were then incubated at 37 °C for 1 h with rat brain cytosol (1 mg/ml protein). The samples were fractionated again into membrane and cytosol fractions, which were analysed by immunoblotting. For the PKC α translocation assay *in vivo*, HeLa cells (5×10^6 per dish) transfected with the indicated plasmids were incubated with or without PI-PLC (0.5 unit) at 37 °C for 1 h or with 1 µM PMA at 37 °C for 20 min. The cells were fractionated into membrane and cytosol fractions, which were analysed by immunoblotting.

Immunoblotting

Proteins separated by SDS/PAGE [7.5 % (w/v) gel] were transferred to a PVDF membrane (Millipore), followed by incubation with the indicated primary antibodies for 1 h. Peroxidase-conjugated anti-rabbit IgG (1:2000 dilution) or mouse IgG (1:1000 dilution) antibodies were used as secondary antibodies. The immunoreactive proteins were detected with the enhanced chemiluminescence kit. The immunoblots were scanned and analysed by NIH Image software as described previously [29].

Immunofluorescence microscopy

Cells grown on glass coverslips were fixed at 25 °C for 10 min with 4 % (w/v) paraformaldehyde, then permeabilized with 0.1 % saponin in PBS for 20 min. Cells were incubated with a combination of mouse monoclonal anti-PKC α (1:100 in PBS) and rabbit polyclonal anti-calnexin (1:300 in PBS) or rabbit polyclonal anti-giantin (1:300 in PBS) for 15 min. After being washed, the cells were stained for 15 min with Fluorolink Cy2-labelled donkey anti-mouse IgG (1:50 in PBS) for PKC α and with rhodamine-conjugated goat anti-rabbit IgG (1:50 in PBS) for calnexin or giantin [29].

Protein phosphorylation

Cells (5×10^6 cells per dish) were labelled at 37 °C for 5 h with [32 P]P_i in 2 ml of Eagle's minimum essential medium lacking phosphate. After being incubated for 30 min with or without 1 μ M PMA, cells were separated from medium and lysed, then subjected to immunoprecipitation with anti-(c-Fos) antibodies in combination with Protein G-Sepharose. When indicated, 32 P-labelled cells were homogenized and separated into cytosol and membrane fractions, followed by immunoprecipitation with anti-PKC α antibodies in combination with Protein A-Sepharose. The immunocomplexes were analysed by SDS/PAGE [7.5% (w/v) gel] and fluorography.

RESULTS

GPI-anchor cleavage by GPI-PLD in the endoplasmic reticulum (ER)

PLAP is a well-characterized GPI-anchored protein [21,22]. We examined the involvement of GPI-PLD in the release of PLAP in HeLa cells that had been transfected with the respective cDNA species. When PLAP was expressed alone, the newly synthesized PLAP remained associated with the cells and was not released into the medium (Figure 1A, panel a). In contrast, when PLAP was co-expressed with GPI-PLD, a substantial amount of PLAP was released into the medium (Figure 1A, panel b), suggesting that the release of PLAP is caused by the exogenously introduced GPI-PLD. In fact, the GPI-PLD expressed by transfection was easily detected by Western blotting and immu-

noprecipitation from [35 S]methionine-labelled cells [18], whereas the level of the endogenous enzyme in HeLa cells was too low to be detected by the same methods (results not shown). It was also found that PLAP was not released from the cells when medium containing GPI-PLD was used for the chase (Figure 1A, panel c). The results suggest that the newly synthesized GPI-PLD is able to cleave the GPI anchor of PLAP only within the cells, not after being secreted.

We then examined a possible intracellular site of GPI-PLD action for GPI cleavage, for which BFA was used to block the transport of the newly synthesized PLAP and GPI-PLD from the ER to the Golgi. When analysed by phase separation with Triton X-114, PLAP that had been expressed alone was recovered exclusively in the detergent phase (Figure 1B, lanes 1 and 2); this was not influenced by the treatment of cells with BFA (Figure 1B, lanes 4 and 5). In contrast, when GPI-PLD was co-expressed in the presence of BFA, PLAP was not secreted (Figure 1B, lane 12) but accumulated as a soluble form that was partitioned into the aqueous phase (Figure 1B, lane 11). The results suggest that PLAP is released from the membrane by GPI-PLD in the ER.

Quantitative determination of DAG

HeLa cells express endogenous GPI-anchored proteins including decay-accelerating factor [30,31]. Cleavage of GPI-anchored proteins by GPI-PLD results in the production of PA in membranes, which is expected to be converted into DAG. We therefore examined whether DAG is increased in cells with or without the expression of GPI-PLD. [32 P]PA converted from DAG in the presence of DAG kinase and [γ - 32 P]ATP was analysed by TLC (Figure 2A). The radioactivity in each PA spot was determined and used to estimate the quantity of DAG (Figure 2B). Control cells without expression of GPI-PLD contained 122 pmol of DAG (Figure 2A, sample 1). When GPI-PLD was expressed, DAG was increased to 264 pmol (Figure 2A, sample 2), which was more than twice that in the control cells. These results suggest that the cleavage of GPI anchors by GPI-PLD induces DAG production. The total potential amount of DAG was also determined by an analysis of cells that had been treated with PI-PLC; it was found not to be significantly changed by expression of GPI-PLD (Figure 2A, samples 3 and 4). Because the direct product of GPI-PLD action is PA, we also tried to detect a difference in PA levels between the two conditions of cells with or without transfection. However, it was quite difficult to detect the difference between the mass levels of PA, possibly owing to its rapid turnover.

Membrane association of PKC α induced by GPI-PLD expression

DAG produced in response to various stimulations is known to activate PKC [32]. One widely used approach to monitoring the activation of PKC is to examine its translocation from the cytosol to membranes [33]. We examined whether GPI-PLD expression causes the membrane association of PKC α , a member of the DAG-dependent PKC family.

For the membrane-association assay of PKC α *in vitro*, we used COS-1 cells that contained no detectable PKC α when analysed by immunoblotting (results not shown). Membranes prepared from COS-1 cells under various conditions were incubated with rat brain cytosol, a source of PKC α . The distribution of PKC α in the membrane and cytosol fractions was analysed by immunoblotting (Figure 3A); the amount of membrane-associated PKC α was normalized against the total PKC α used (Figure 3D). The amount of PKC α associated with membranes, the basal level of which was approx. 20% (Figures 3A and 3D, mock), was increased to approx. 50% in the PI-PLC-treated membranes

Figure 1 Intracellular release of PLAP by GPI-PLD

(A) HeLa cells expressing PLAP alone (a, c) or both PLAP and GPI-PLD (b) were pulse-labelled with [35 S]methionine for 30 min and chased in complete fresh medium (a, b) or in complete medium containing GPI-PLD (c). At the indicated times of chase, cell lysates and media were prepared and used for immunoprecipitation with anti-PLAP. The immunoprecipitates were analysed by SDS/PAGE [7.5% (w/v) gel] and fluorography. (B) Cells expressing PLAP alone (PLAP) or both PLAP and GPI-PLD (PLAP/PLD) were pulse-labelled and chased for 3 h in the presence (+) or absence (–) of 5 μ g/ml BFA. The cells were separated by phase separation with Triton X-114 into detergent (Dt) and aqueous (Aq) phases. PLAP in each phase and in culture medium (M) was immunoprecipitated and analysed as above. Apparent molecular masses (in kDa) are indicated at the right.

Figure 2 Quantitative determination of DAG

Control cells (samples 1 and 3) and cells expressing GPI-PLD (samples 2 and 4) were homogenized in PBS. One half of the homogenate was used directly for lipid extraction (samples 1 and 2) and the other half was incubated at 37 °C for 1 h with PI-PLC before extraction (samples 3 and 4). The lipid extracts were analysed with the DAG assay reagent system and TLC, followed by the quantification of DAG as described in the Materials and methods section. **(A)** An autoradiogram of ^{32}P -labelled PA on the TLC plate. **(B)** The amount of DAG calculated from the radioactivity of each PA spot in **(A)**. The values (pmol per sample) are means $\pm$ S.D. ($n = 3$).

(Figures 3A and 3D, PLC) and to approx. 60% in the membranes from GPI-PLD-expressing cells with or without co-expression of PLAP (Figures 3A and 3D, PLD and PLAP/PLD). PKC α was almost completely associated with the membranes by treatment with PMA, which is known to activate the kinase as a DAG analogue (Figures 3A and 3D, PMA).

The membrane association of endogenous PKC α *in vivo* was determined by using HeLa cells (Figures 3B and 3E). The basal level of PKC α associated with membranes was less than 40% of the total PKC α and was not influenced by the expression of PLAP alone (Figures 3B and 3E, mock and PLAP). In addition, in contrast with the assay *in vitro*, treatment of cells with PI-PLC did not cause an increase in translocation (Figures 3B and 3E, PLC), suggesting that PI-PLC could not attack phosphatidylinositol, which is a main source for DAG generation and is localized in the inner leaflet of the plasma membrane. However, the expression of GPI-PLD greatly increased the translocation of PKC α to the membranes (Figures 3B and 3E, PLD) but this effect was not as great when GPI-PLD was co-expressed with PLAP (Figures 3B and 3E, PLAP/PLD). PLAP is anchored to the membrane by alkylacylglycerol, not by DAG [34,35]; alkylacylglycerol neither stimulates PKC nor inhibits its activation by DAG [36], as observed in the assay *in vitro* with or without PLAP expression (Figures 3A and 3D, PLD and PLAP/PLD). The decreased level of translocation observed by co-expression with PLAP in the assay *in vivo* could be explained by the possibility

Figure 3 Membrane association of PKC α *in vitro* and *in vivo*

(A) Membranes were prepared from control COS-1 cells (mock) and from cells expressing PLAP, GPI-PLD (PLD) or both PLAP and GPI-PLD (PLAP/PLD) and the control cell membranes were treated with PI-PLC (PLC) or PMA, as described in the Materials and methods section. The membranes were then incubated at 37 °C for 1 h with rat brain cytosol (1 mg/ml protein). The samples were again fractionated into membranes (M) and cytosol supernatants (Cy), which were analysed by immunoblotting with anti-PKC α antibodies. **(B)** Membrane (M) and cytosol (Cy) fractions were prepared from control HeLa cells before (mock) or after treatment with PI-PLC (PLC) or PMA and from cells expressing PLAP, GPI-PLD (PLD) or both PLAP and GPI-PLD (PLAP/PLD). The samples were analysed by immunoblotting as above. **(C)** Control HeLa cells (mock/PLD) were incubated in medium containing GPI-PLD and then fractionated into membranes (M) and cytosol (Cy), followed by immunoblotting. The amount of PKC α in each fraction was determined and its recovery in the membrane fraction is expressed as a percentage of the total PKC α (membrane plus cytosol) (mean $\pm$ S.D.; $n = 3$). The data shown in **(A)** are represented in **(D)**; those in **(B)** and **(C)** are summarized in **(E)**.

that the overexpressed PLAP might be competitive for the hydrolysis by GPI-PLD of endogenous GPI-anchored proteins containing DAG. It was also confirmed that the incubation of cells in the medium containing GPI-PLD did not cause such an increase in the translocation of PKC α (Figures 3C and 3E, mock/PLD). These results suggest that the translocation of PKC α to the membranes is induced by the intracellular GPI-PLD expressed by the transfection, possibly through the cleavage of GPI moieties.

Subcellular localization of PKC α

We then examined the subcellular localization of PKC α : a postnuclear fraction of HeLa cells was centrifuged through a linear or stepwise sucrose density gradient, then fractionated and

Figure 4 Subcellular distribution of PKC α analysed by sucrose-density-gradient centrifugation

Postnuclear fractions were prepared from HeLa cells before (a–e) or after treatment with PMA (f) and from cells expressing GPI-PLD (g) and centrifuged on a continuous (A) or stepwise (B) sucrose density gradient, as described in the Materials and methods section; 17 fractions (A) or 5 fractions (B) were collected and analysed by immunoblotting with antibodies against ARF, giantin, calnexin, integrin β 3 and PKC α .

analysed by immunoblotting (Figure 4). Fractions obtained from the linear sucrose gradient were characterized for the distribution of marker proteins. ARF, which is present primarily in the cytosol and partly localized to the Golgi, was enriched mostly in fractions 15–17 and detected weakly in fractions 1 and 2 (Figure 4A, panel a). The Golgi marker giantin [24] was enriched in fractions 1 and 2 (Figure 4A, panel b). The ER marker calnexin [23] was also enriched in fractions 1 and 2 (Figure 4A, panel c). The plasma membrane marker integrin β 3 was distributed mainly in fractions 3–9 (Figure 4A, panel d). We therefore conclude that fractions 1 and 2 contain the ER and the Golgi, whereas fractions 3–9 and 15–17 contain the plasma membrane and the cytosol respectively. When analysed under these conditions, PKC α from the control cells was distributed mainly in the cytosol (fractions 15–17), although faintly detectable in the plasma membrane (fractions 3–9) (Figure 4A, panel e). In contrast, on treatment of cells with PMA, most PKC α was distributed in the plasma membrane fraction (Figure 4A, panel f). The expression of GPI-PLD also caused a marked change in the distribution of PKC α : most of the kinase was recovered in fractions 1 and 2 (the ER and Golgi), although it was detected weakly in the plasma membrane and cytosol fractions (Figure 4A, panel g). This profile is quite different from that of the PMA-treated cells.

Better resolution of the ER from the Golgi was obtained by centrifugation through a stepwise sucrose gradient (Figure 4B): fractions 1 and 2 contained the ER, fraction 3 contained the Golgi, and fractions 4 and 5 contained the plasma membrane and the cytosol respectively. PKC α from the control cells (Figure 4B, panel e) and PMA-treated cells (Figure 4B, panel f) was distributed mainly in the combined cytosol and plasma membrane fractions. When GPI-PLD was expressed, PKC α was recovered mainly in the ER fractions, although it was also detected at a lower level in the Golgi fraction (Figure 4B, panel g).

The intracellular distribution of PKC α was also examined by immunofluorescence microscopy (Figure 5). Control cells were stained diffusely, indicating the cytosolic distribution of PKC α (Figure 5a). Treatment of cells with PMA increased the staining of the cell periphery, suggesting the localization of PKC α to the plasma membrane (Figure 5b). When GPI-PLD was expressed,

Figure 5 Intracellular localization of PKC α analysed by immunofluorescence microscopy

HeLa cells before (a) or after treatment with PMA (b) and cells expressing GPI-PLD (c–f) were fixed, permeabilized and immunostained as described in the Materials and methods section. The cells in (a) and (b) were immunostained for PKC α ; those in (c) and (d) were double-stained for PKC α and calnexin respectively; and those in (e) and (f) were also double-stained for PKC α and giantin respectively. Scale bar, 10 μ m.

PKC α was more concentrated in perinuclear regions (Figures 5c and 5e) and mostly co-localized with the ER marker calnexin, as shown when the cells were double-stained (Figures 5c and 5d). However, this staining pattern was clearly different from that for the Golgi marker giantin (Figures 5e and 5f). Taken together, these results suggest that the expression of GPI-PLD induces the translocation of PKC α to the ER.

Figure 6 Phosphorylation of PKC α and c-Fos

Control HeLa cells (mock) and cells expressing GPI-PLD (PLD) were labelled with [32 P]P $_i$ for 5 h; the control cells were further incubated for 30 min in the absence (mock) or presence of 1 μ M PMA. (A) Cells were homogenized and fractionated into membranes (M) and cytosol (Cy), followed by immunoprecipitation with anti-PKC α (upper panel) or by immunoblotting (lower panel). (B) Cell lysates were prepared and used for immunoprecipitation with anti-(c-Fos). The immunoprecipitates were analysed by SDS/PAGE [7.5% (w/v) gel] and autoradiography.

Protein phosphorylation

It is well known that translocation of PKC from the cytosol to membranes involves the activation of PKC [33], resulting in its autophosphorylation [37] and the phosphorylation of other substrates such as c-Fos [38]. In fact, when cells were labelled with [32 P]P $_i$, all the 32 P-labelled PKC α was recovered in the membrane fraction (Figure 6A, lanes 1, 3 and 5). The expression of GPI-PLD increased the phosphorylation of PKC α markedly, to a level similar to that obtained by the treatment of cells with PMA (Figure 6A, lanes 3 and 5). We also examined the phosphorylation of c-Fos, a substrate phosphorylated by PKC. Its phosphorylation was greatly enhanced in cells expressing GPI-PLD (Figure 6B, lane 1) as well as in cells treated with PMA (Figure 6B, lane 2), in comparison with that of the control cells (Figure 6B, lane 1). Taken together, these results strongly suggest that the expression of GPI-PLD is involved in the intracellular translocation and phosphorylation/activation of PKC α .

DISCUSSION

GPI-PLD is abundantly present in serum and has unique catalytic properties: the enzyme readily hydrolyses GPI moieties from detergent-solubilized GPI-anchored proteins but it is not active towards these substrates when anchored to intact membranes [17,18], in contrast with PI-PLC, which hydrolyses both the detergent-solubilized and intact membrane proteins. Therefore its site of action and functions *in vivo* have remained unclear. Two possible intracellular sites of GPI-PLD action have been proposed. The enzyme, possibly taken up from serum, was enriched in a lysosomal fraction purified from rat liver and hydrolysed the GPI moieties of GPI-anchored proteins [39]. It was also suggested that the enzyme in the secretory pathway is involved in the cleavage of the GPI anchor [31]. This was confirmed by transfection experiments: when co-expressed with GPI-PLD, GPI-anchored proteins are intracellularly hydrolysed

by the newly synthesized GPI-PLD [18–20]. In fact, as demonstrated in the present study, the GPI-anchored protein PLAP was released intracellularly from the membrane by the co-expressed GPI-PLD and secreted as a soluble form into the medium. The results obtained by treatment with BFA support the possibility that the cleavage of GPI by GPI-PLD occurs in the ER. It was suggested that an immature form of GPI-PLD in the ER is conformationally different from the mature secreted form and is important to its action [18]. The enzyme found in lysosomes might also undergo a conformational change caused by other hydrolases, including sialidase.

In contrast, it has been suggested that GPI-anchored proteins are involved in signal transduction directly or indirectly through its derivatives PA or DAG, although it remained unclear which enzyme is involved in the production of DAG or PA from GPI-anchored proteins [11–13]. To clarify that the PA or DAG production is indeed induced by GPI-PLD action, we analysed the levels of DAG. In response to GPI-PLD expression the DAG level was more than doubled compared with that of control cells. Because this value was obtained with whole membranes, its increase in the ER membrane might be greater than this. It is also likely that PA produced by GPI-PLD is rapidly converted into DAG through the action of PA phosphatase [40]. This prolonged accumulation of DAG is comparable to other observations that the persistent elevation of DAG concentration is generated by the sequential actions of PLD and PA phosphatase in several systems [41–43]. We therefore suggest that GPI-PLD substrates in the ER, GPI-anchored proteins or GPI precursors, are new potential sources of DAG production.

On the basis of these findings we examined whether the GPI-PLD-dependent accumulation of DAG could induce the activation of PKC. PKC exists as a family of at least 12 isoenzymes with closely related structures [44]. All identified PKCs share a common functional requirement for phosphatidylserine and differ in their sensitivities to other activators [44]. Conventional PKCs, including the four isotypes α , β I, β II and γ , are activated by Ca $^{2+}$ and DAG (which are increased by a variety of physiological stimulators such as hormones and growth factors) or by non-physiological stimulators, including phorbol esters such as PMA [32,45]. It is also known that PKCs are translocated from the cytosol to the cell membrane when activated [32,45]. In the present study we demonstrated that an increased amount of PKC α was associated with membranes, in particular with the ER membrane, in cells overexpressing GPI-PLD. Although most PKC α was localized to the plasma membrane when cells were treated with PMA, it is interesting that a significant amount of PKC α was also associated with the ER membrane, which is consistent with results obtained for NIH 3T3 cells [46]. Dobberstein and co-workers [47] recently reported that ER membrane proteins of the translocation machinery (Sec61 β , docking protein α and TRAMP) can be phosphorylated in a Ca $^{2+}$ -dependent manner and that PKC isoforms α and β are associated with the ER membrane, suggesting that the translocation of newly synthesized proteins across the ER membrane is regulated by PKCs. In addition, several other studies have suggested the involvement of PKCs in the phosphorylation of proteins that regulate vesicular transport [48–50]. These include Sec31p, a component of the ER export machinery [48], COP I, a complex of coat proteins [49], and ARF, a GTPase involved in the binding of COP I to the Golgi membrane [50]. Taken together, these studies suggest that the early stage in the secretory pathway is regulated by PKCs. It is therefore likely that PKC α , which is redistributed to and activated on the ER membrane in response to the cleavage of GPI moieties, might also be involved in the regulation of the early secretory pathway. Further studies

will be required to elucidate the target(s) of PKC α activated under our conditions.

In contrast, it has been shown that a membrane-associated PLD exerts an essential role in membrane trafficking through the ER [51] and the Golgi complex [40,52], and that the activation of PLD is itself mediated by PKCs [53]. Therefore the activation of PKC α on the ER membrane through the action of GPI-PLD might result in the further activation of PKC-dependent PLD. In addition, PA, which is a direct product of PLD, has been proposed to participate in the regulation of vesicular transport in several systems [40,54]. Therefore we cannot exclude the possibility that PA, itself generated by GPI-PLD, might be an important factor in cellular events, although we have no direct evidence for this at present.

The involvement of GPI-PLD in the regulation of PKC is demonstrated here in cells overexpressing GPI-PLD, raising a question about the physiological significance of these findings. The available evidence indicates that the hydrolysis of GPI is related to signal transduction. Various hormones and cytokines induce the hydrolysis of GPI and the inositolphosphoglycans produced, as well as DAG, act as second messengers [11–13]. However, there has been no direct evidence demonstrating that these stimulators also induce the expression of GPI-PLD. It is clear that despite being at a high level, the serum-derived GPI-PLD is not involved in the event because the serum PLD cannot cleave GPI on the intact membrane. In our previous study [18] we showed that GPI-PLD is expressed only in liver and brain. This suggests the possibility that PKC is activated through the expression of GPI-PLD only in these tissues. It is of interest that brain is a tissue enriched in PKC activity and GPI-anchored proteins. In addition, GPI-anchored proteins such as carcino-embryonic antigen ('CEA') are released into serum in some malignant tumours [55]. It will therefore also be of interest to examine whether GPI-PLD is expressed at a high level and PKC is activated in these tissues.

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REFERENCES

- Lisanti, M. P., Sargiacomo, M., Graeve, L., Saltiel, A. R. and Rodriguez-Boulton, E. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 9557–9561
- Lisanti, M. P., Scherer, P. E., Vidugiriene, J., Tang, Z., Hermanowski-Vosatka, A., Tu, Y. H., Cook, R. F. and Sargiacomo, M. (1994) *J. Cell Biol.* **126**, 111–126
- Schnitzer, J. E., McIntosh, D. P., Dvorak, A. M., Liu, J. and Oh, P. (1995) *Science* **269**, 1435–1439
- Brown, D. A. and Rose, J. K. (1992) *Cell* **68**, 533–544
- Hope, H. R. and Pike, L. J. (1996) *Mol. Biol. Cell* **7**, 843–851
- Arreaza, G., Melkonian, K. A., LaFevre-Bernt, M. and Brown, D. A. (1994) *J. Biol. Chem.* **269**, 19123–19127
- Smart, E. J., Ying, Y. S. and Anderson, R. G. (1995) *J. Cell Biol.* **131**, 929–938
- Oka, N., Yamamoto, M., Schwencke, C., Kawabe, J., Ebina, T., Ohno, S., Couet, J., Lisanti, M. P. and Ishikawa, Y. (1997) *J. Biol. Chem.* **272**, 33416–33421
- Stefanova, I., Horejsi, V., Anstegui, I. J., Knapp, W. and Stockinger, H. (1991) *Science* **254**, 1016–1019
- Stefanova, I., Corcoran, M. L., Horak, E. M., Wahl, L. M., Bolen, J. B. and Horak, I. D. (1993) *J. Biol. Chem.* **268**, 20725–20728
- Chan, B. L., Chao, M. V. and Saltiel, A. R. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 1756–1760
- Merida, I., Pratt, J. C. and Gaulton, G. N. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 9421–9425
- Represa, J., Avila, M. A., Miner, C., Giraldez, F., Romero, G., Clemente, R., Mato, J. M. and Varela-Nieto, I. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8016–8019
- Tachado, S. D., Gerold, P., Schwarz, R., Novakovic, S., McConville, M. and Schofield, L. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94**, 4022–4027
- Deeg, M. A. and Davitz, M. A. (1995) *Methods Enzymol.* **250**, 630–640
- Davitz, M. A., Herald, D., Shak, S., Krakow, J., Englund, P. T. and Nussenzweig, V. (1987) *Science* **238**, 81–84
- Low, M. G. and Huang, K. S. (1991) *Biochem. J.* **279**, 483–493
- Tsujioka, H., Misumi, Y., Takami, N. and Ikehara, Y. (1998) *Biochem. Biophys. Res. Commun.* **251**, 737–743
- Scallan, B. J., Fung, W. J., Tsang, T. C., Li, S., Kado-Fong, H., Huang, K. S. and Kochan, J. P. (1991) *Science* **252**, 446–448
- Bernasconi, E., Fasel, N. and Wittek, R. (1996) *J. Cell Sci.* **109**, 1195–1201
- Takami, N., Ogata, S., Oda, K., Misumi, Y. and Ikehara, Y. (1988) *J. Biol. Chem.* **263**, 3016–3021
- Ogata, S., Hayashi, Y., Takami, N. and Ikehara, Y. (1988) *J. Biol. Chem.* **263**, 10489–10494
- Wada, I., Ou, W. J., Liu, M. C. and Scheele, G. (1994) *J. Biol. Chem.* **269**, 7464–7472
- Sohda, M., Misumi, Y., Fujiwara, T., Nishioka, M. and Ikehara, Y. (1994) *Biochem. Biophys. Res. Commun.* **205**, 1399–1408
- Bordier, C. (1981) *J. Biol. Chem.* **256**, 1604–1607
- Bligh, E. A. and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917
- Lewis, V. A., Hynes, G. M., Zheng, D., Saibil, H. and Willson, K. (1992) *Nature (London)* **358**, 249–252
- Taylor, G. A., Stauber, R., Rulong, S., Hudson, E., Pei, V., Pavlakis, G. N., Resau, J. H. and Vande Woude, F. G. (1997) *J. Biol. Chem.* **272**, 10639–10645
- Sohda, M., Misumi, Y., Yano, A., Takami, N. and Ikehara, Y. (1998) *J. Biol. Chem.* **273**, 5385–5388
- Medof, M. E., Walter, E. I., Rutgers, J. L., Knowles, D. M. and Nussenzweig, V. (1987) *J. Exp. Med.* **165**, 848–864
- Metz, C. N., Brunner, G., Choi-Muir, N. H., Nguyen, H., Gabrilove, J., Caras, I. W., Altszuler, N., Rifkin, D. B., Wilson, E. L. and Davitz, M. A. (1994) *EMBO J.* **13**, 1741–1751
- Nishizuka, Y. (1986) *Science* **233**, 305–312
- Pelech, S. L., Meier, K. E. and Krebs, E. G. (1986) *Biochemistry* **25**, 8348–8353
- Redman, C. A., Thomas-Oates, J. E., Ogata, S., Ikehara, Y. and Ferguson, M. A. (1994) *Biochem. J.* **302**, 861–865
- Brewis, I. A., Ferguson, M. A., Mehler, A., Turner, A. J. and Hooper, N. M. (1995) *J. Biol. Chem.* **270**, 22946–22956
- Daniel, L. W., Huang, C., Strum, J. C., Smitherman, P. K., Greene, D. and Wykle, R. L. (1993) *J. Biol. Chem.* **268**, 21519–21526
- Wolf, M., Cuatrecasas, P. and Sahyoun, N. (1985) *J. Biol. Chem.* **260**, 15718–15722
- Barber, J. R. and Verma, I. M. (1987) *Mol. Cell. Biol.* **7**, 2201–2211
- Hari, T., Kunze, H., Bohn, E., Brodbeck, U. and Butikofer, P. (1996) *Biochem. J.* **320**, 315–319
- Singer, W. D., Brown, A. and Sternweis, P. C. (1997) *Annu. Rev. Biochem.* **66**, 475–509
- Billah, M. M. and Anthes, J. C. (1990) *Biochem. J.* **269**, 281–291
- Exton, J. H. (1994) *Biochim. Biophys. Acta* **1212**, 26–42
- Cesnjaj, M., Zheng, L., Catt, K. J. and Stojilkovic, S. S. (1995) *Mol. Biol. Cell* **6**, 1037–1047
- Tanaka, C. and Nishizuka, Y. (1994) *Annu. Rev. Neurosci.* **17**, 551–567
- Blumberg, P. M. (1988) *Cancer Res.* **48**, 1–8
- Goodnight, J. A., Mischak, H., Kolch, W. and Mushinski, J. F. (1995) *J. Biol. Chem.* **270**, 9991–10001
- Gruss, O. J., Feick, P., Frank, R. and Dobberstein, B. (1999) *Eur. J. Biochem.* **260**, 785–793
- Salam, N. R., Chuang, J. S. and Shekman, R. W. (1997) *Mol. Biol. Cell* **8**, 205–217
- Sheff, D., Lowe, M., Kreis, T. E. and Mellmann, I. (1996) *J. Biol. Chem.* **271**, 7230–7236
- De Matteis, M. A., Santini, G., Kahn, R. A., Di Tullio, G. and Luini, A. (1993) *Nature (London)* **364**, 818–821
- Decker, C., Miro, O. H., Silence, D. J. and Allan, D. (1996) *Biochem. J.* **320**, 855–890
- Kahn, R. A., Yucel, J. K. and Malhotra, V. (1993) *Cell* **75**, 1045–1048
- Hammond, S. M., Jenco, J. M., Nakashima, S., Cadwallader, K., Gu, Q., Cook, S., Nozawa, Y., Prestwich, G. D., Frohman, M. A. and Morris, A. J. (1997) *J. Biol. Chem.* **272**, 3860–3868
- Williger, B. T., Ho, W. T. and Exton, J. H. (1999) *J. Biol. Chem.* **274**, 735–738
- Kuroki, M., Arakawa, F., Yamamoto, H., Shimura, H., Ikehara, Y. and Matsuoka, Y. (1988) *Cancer Lett.* **43**, 151–157