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Phosphorylation and redistribution of the phosphatidylinositol-transfer protein in phorbol 12-myristate 13-acetate- and bombesin-stimulated Swiss mouse 3T3 fibroblasts

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By immunofluorescence microscopy it was shown that the phosphatidylinositol-transfer protein (PI-TP) becomes associated with the Golgi membranes when confluent (quiescent) Swiss mouse 3T3 fibroblast cells are stimulated with phorbol 12-myristate 13-acetate (PMA) and bombesin. Dibutyryl cyclic AMP or dexamethasone had no effect on the intracellular redistribution of PI-TP. In exponentially growing cells and in serum-starved (semi-quiescent) cells, PI-TP is already associated with Golgi structures. Stimulation of semi-quiescent cells by PMA resulted in a rapid redistribution of PI-TP. A similar yet slower response was observed after stimulation with bombesin. Stimulation of semi-quiescent 3T3 cells by PMA significantly increased the phosphorylation of PI-TP, as shown by immunoprecipitation of PI-TP from pre-labelled cells. No significant increase in phosphorylation of PI-TP was observed after stimulation of these cells by bombesin. Purified PI-TP was shown to be a substrate for protein kinase C *in vitro*. The possibility that the phosphorylation of PI-TP after activation of protein kinase C is involved in the observed redistribution of PI-TP is discussed.

INTRODUCTION

Proteins which are able to catalyse the transfer of phospholipid molecules between membranes have been purified and characterized from a wide spectrum of cells and tissues (reviewed in [1]). Phospholipid-transfer proteins with different specificities toward the polar headgroups of phospholipids include the phosphatidylcholine (PC)-transfer protein, which specifically binds and transfers PC [2,3], and the phosphatidylinositol-transfer protein (PI-TP) which binds and transfers PI, and to a lesser extent PC [4–6]. In addition, a non-specific lipid-transfer protein (identical with sterol carrier protein 2) has been identified, which catalyses the transfer of phospholipids and cholesterol between membranes [7,8]. The characteristics of these proteins have been studied extensively *in vitro*. However, the physiological function of the phospholipid-transfer proteins is not yet clear.

In mammalian cells most phospholipids are synthesized on the endoplasmic reticulum. This implies that specific mechanisms of transport must operate to redistribute phospholipids from the site of synthesis to the proper localization in the cell. It has been suggested that PI-TP is involved in this transport process, particularly pertaining to those membranes that have an active PI metabolism [9–11]. Presumably, transfer of PI to these membranes occurs by PI-TP directly or by a flow of membrane vesicles, the PI content of which has been modulated by PI-TP. Recently, the interest in the physiological role of PI-TP has strongly increased with the observation that PI-TP in yeast is identical with the SEC14 protein. This protein is involved in secretory processes at the level of the late Golgi compartment [12,13]. Localization studies showed that PI-TP in yeast is associated with Golgi structures. Furthermore, it was shown that deletion of the gene encoding PI-TP in yeast is lethal for the organism [14]. The requirement for PI-TP can be by-passed by a mutation in the CDP-choline pathway for PC biosynthesis [11,15]. These studies have been interpreted to indicate that PI-TP has the capacity to control the phospholipid composition of the yeast Golgi membranes, which is proposed to be critical to the secretory competence of these membranes.

In systems *in vitro*, both yeast and mammalian PI-TP express a similar capacity to bind PI or PC and transfer PI or PC between membranes. In addition, both proteins have the same molecular mass and a similar isoelectric point. On the other hand, mammalian and yeast PI-TP lack amino acid sequence similarity [13,16] as well as immunological cross-reactivity [17]. Recently, we have shown that in mouse 3T3 fibroblast cells PI-TP is present in the cytoplasm and is associated with the Golgi system [18]. Furthermore, it was shown that in metabolically active (i.e. exponentionally growing) cells, the amount of Golgi-associated and cytoplasmic PI-TP is significantly increased as compared with quiescent (either confluent or serum-starved) cells. These results suggested that, similar to what is observed in yeast cells, PI-TP also fulfils a role in the functioning of the Golgi system in mammalian cells.

In order to gain a better understanding of the factors that control the association of PI-TP with the Golgi system, we have studied this protein in quiescent (confluent) and in semi-quiescent (serum-starved) Swiss mouse 3T3 fibroblast cells under conditions of agonist-induced activation. It is shown below that upon activation with phorbol 12-myristate 13-acetate (PMA) and bombesin [19–24] PI-TP becomes redistributed. Evidence is presented that activation of semi-quiescent 3T3 cells by PMA leads to phosphorylation of PI-TP and that *in vitro* PI-TP is a substrate for protein kinase C.

MATERIALS AND METHODS

Materials

Protein A–Sepharose, bombesin, leupeptin, aprotinin, ATP,

Abbreviations used: PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; PI-TP, PI-transfer protein; DAG, diacylglycerol; FCS, fetal-calf serum; PMA, phorbol 12-myristate 13-acetate; PBS0, phosphate-buffered saline without Ca^{2+} and Mg^{2+}; FITC, fluorescein isothiocyanate; TRITC, tetramethyl rhodamine isothiocyanate; DMEM, Dulbecco's Minimum Essential Medium.

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tetramethyl rhodamine isothiocyanate (TRITC)–ricin (TRITC-Toxin RCA60) and PMA were obtained from Sigma. Phosphatidylserine (PS) was from Lipid Products, South Nutfield, Surrey, U.K. Fluorescin isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (GAR) was from Nordic Immunological Laboratories, Tilburg, The Netherlands. [γ-^{32}P]ATP (3000 Ci/mmol), [^{35}S]methionine (1136 Ci/mmol) and Hyperfilm-MP were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Dulbecco's modified Eagle's medium (DMEM) and DF medium were obtained from Gibco BRL. Soluene-350 and Insta-Fluor were from Packard, Downers Grove, IL, U.S.A. Mowiol was a gift from Hoechst.

Cell culture

Swiss mouse 3T3 fibroblast cells were cultured in DMEM containing 7.5% fetal-calf serum (FCS) and buffered with $NaHCO_3$ (44 mM) at 37 °C in a humidified atmosphere with 7.5% CO_2. Cells were made quiescent by density arrest. Cell cultures were used 7–10 days after reaching confluency, and the medium was changed at 3-day intervals [25]. To obtain serum-starved (semi-quiescent) cells the normal growth medium was removed 2 h after trypsin treatment and seeding of the cells. The cells were cultured for 2 days in DMEM containing 0.5% FCS [26].

Immunofluorescence

The localization of PI-TP was determined by indirect immunofluorescence using the polyclonal anti-PI-TP antibody raised against synthetic peptides representing the amino acid sequences of predicted epitopes in rat brain PI-TP [16,18]. Cells grown on glass coverslips were fixed with methanol at −20 °C for 4 min. The cells were then rinsed with phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS0) and incubated with DMEM, buffered with Hepes (25 mM) and containing 0.1% BSA (DBH), for 30 min at room temperature to block non-specific binding sites. The cells were exposed to the anti-PI-TP antibody or to control IgG isolated from pre-immune serum (both diluted 1:100 with DBH) for 1 h at room temperature. The cells were rinsed with PBS0, incubated for 1 h at room temperature with GAR–FITC (diluted 1:80 with DBH), rinsed with PBS0 and mounted in mowiol. The Golgi system was made visible by labelling the fixed cells with TRITC–ricin (1:60 diluted with DBH). The labelled cells were viewed with a Leitz inverted microscope.

Purification of protein kinase C and PI-TP

Protein kinase C was purified from rat brain by a modified procedure previously described by Huang et al. [27]. Fresh rat brains (20–40 g of tissue) were homogenized and the cytosolic fraction was subsequently purified on DEAE-Sepharose, Sephacryl 200 and phenyl-Sepharose columns. The purified enzyme has a specific activity of 200 nmol of phosphate/min per mg of protein when assayed with histone IIIs as substrate. The purified enzyme is stable for several months when kept at −40 °C in 50% glycerol and 0.01% Triton X-100. PI-TP was purified from fresh bovine brain as described by Van Paridon et al. [28].

Phosphorylation of PI-TP *in vitro* by protein kinase C

PI-TP (1–3 μg) was phosphorylated in a reaction volume of 125 μl containing 20 mM Tris/HCl, pH 7.5, 7.5 mM magnesium acetate, 10 μg/ml leupeptin, 10 μM ATP, 1 μCi of [γ-^{32}P]ATP. The Ca^{2+}/phospholipid-independent phosphorylation was determined in the presence of 1 mM EGTA, and the Ca^{2+}/phospholipid-dependent phosphorylation was determined in the presence of 1 mM Ca^{2+}, 96 μg/ml PS and 3.2 μg/ml diacylglycerol (DAG). The mixture was incubated for 20 min at 30 °C and the reaction was terminated by addition of trichloroacetic acid to a final concentration of 10% (v/v). Precipitated protein was spun down, dissolved in sample buffer {125 mM Tris/HCl, pH 6.8, 5% (w/v) SDS, 12.5% (v/v) 2-mercaptoethanol and 10% (v/v) glycerol [29]} and analysed by SDS/PAGE (15% gel) and by autoradiography. Phosphorylation of PI-TP as a function of time and concentration was determined under similar conditions.

Phosphorylation of PI-TP *in vivo* and immunoprecipitation

3T3 cells were cultured in DMEM containing 7.5% FCS and buffered with $NaHCO_3$ (44 mM) under a 7.5%-CO_2 atmosphere. Confluent cells in 25 cm^2 flasks were labelled for 5 h with 0.5 mCi of carrier-free [^{32}P]P_i in 2 ml of phosphate-free Ham's F12 (DF) medium containing 5% FCS. In one flask PMA was present during the last 10 min of the labelling. Cell cultures were washed twice with PBS0 containing 10 mM sodium pyrophosphate, scraped in 0.75 ml of lysis buffer [20 mM Hepes, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 2 mM EGTA, 1.5 mM $MgCl_2$, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM phenylmethanesulphonyl fluoride, 10 mM NaF, 10 mM sodium pyrophosphate] and homogenized in the reaction tubes with a tight-fitting Teflon pestle. The homogenates were centrifuged for 10 min at 14000 *g* at 4 °C. Trichloroacetic acid was added to the supernatant to a final concentration of 10% (v/v). The precipitated proteins were resuspended in a small volume of 1 M NaOH, neutralized with an equal volume of 1 M Tris/HCl, pH 6.5. The proteins were denatured by addition of 1 vol. of denaturation buffer (62.5 mM Tris, pH 6.5, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol) and heating at 95 °C for 5 min. The reaction mixture was diluted 10–15-fold with lysis buffer without NaF and sodium pyrophosphate to decrease the SDS concentration. Control IgG (purified from pre-immune serum by using Protein A–Sepharose; 12 μg/incubation) was added and the mixture was rotated overnight at 4 °C. Control IgG was removed with Protein A–Sepharose (3 mg/incubation). Affinity-purified anti-PI-TP antibody (9 μg/incubation) was added to the supernatant, the mixture was rotated overnight at 4 °C and the PI-TP–antibody complex was isolated with Protein A–Sepharose (3 mg/incubation). All Protein A–Sepharose pellets were washed with 3 × 1 ml of 20 mM Hepes, pH 7.4, 150 mM NaCl, 0.05% (v/v) Triton X-100, 10% glycerol (HNTG buffer) and prepared for SDS/PAGE by heating in sample buffer for 5 min at 95 °C. Samples were separated on 15%-acrylamide gels and analysed by autoradiography. The stoichiometry of the phosphorylation *in vivo* was determined as described in [30]. Cell cultures were labelled for 20 h with 25 μCi of [^{35}S]methionine in Hepes-buffered DMEM, pH 7.5, without methionine, but containing 5% FCS. Cell lysates and immunoprecipitates were prepared and analysed as described above. A duplicate dish was used to determine the protein content in the cell lysate by the Lowry method [31]. A third dish of cells was incubated with [^{32}P]P_i for 5 h; the cell lysate and immunoprecipitate of PI-TP were prepared as described above. From the results of the three parallel experiments, the stoichometry of phosphorylation of PI-TP *in vivo* could be calculated.

Gel electrophoresis and elution of proteins from the gels

Samples were analysed by electrophoresis on 15% polyacrylamide gels in the presence of SDS and 2-mercapto-

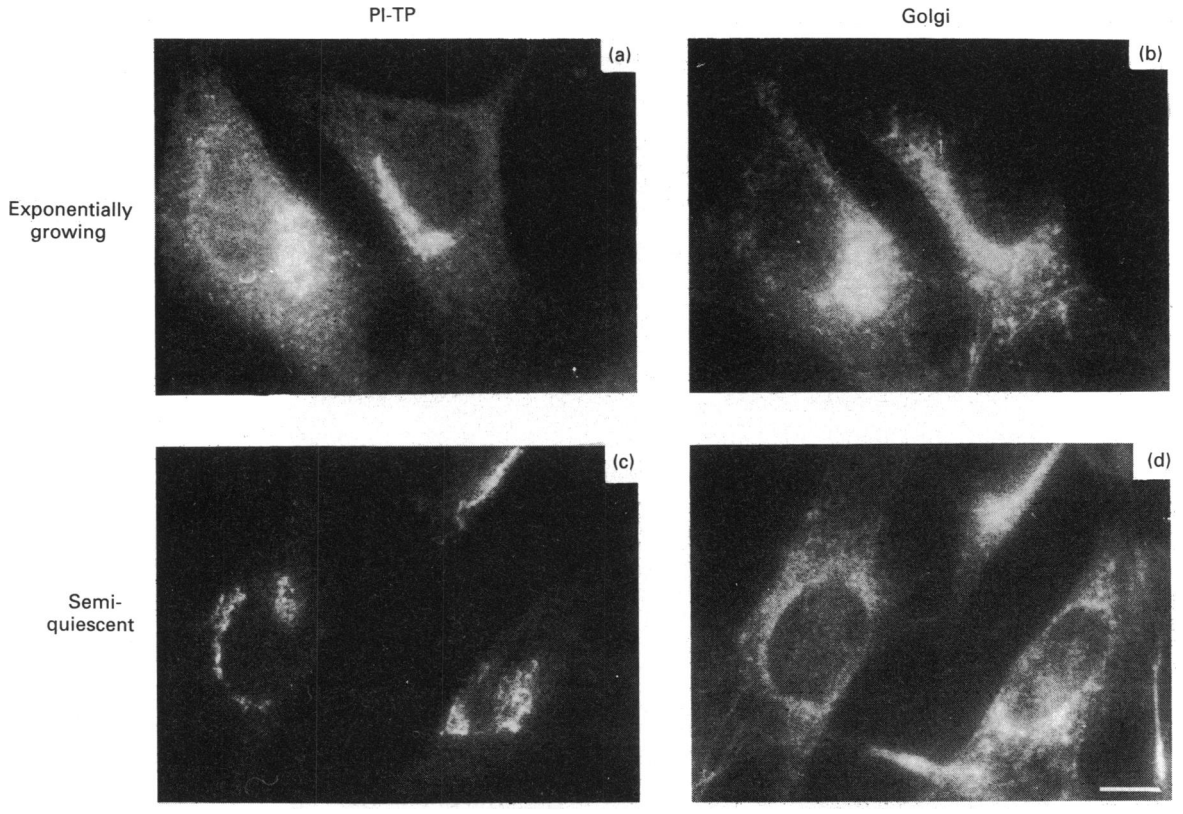

Figure 1 Immunofluorescence localization of PI-TP (a, c) and of sugar groups which are specifically processed in the Golgi system (b, d) in exponentionally growing (a, b) and semi-quiescent (c, d) Swiss mouse 3T3 cells

Cells were grown, made semi-quiescent and fixed as described in the Materials and methods section. Bar: 5 μm.

ethanol as described by Laemmli [29]. Proteins were detected by staining with Coomassie Brilliant Blue. Phosphorylated proteins were detected by autoradiography. Gels were dried and enclosed with one intensifying screen. Hyperfilm MP was used and exposed for 1–48 h at −50 °C. Labelled proteins were eluted from dried gel slices by incubation in 0.1 ml of water and 0.4 ml of Soluene-350 overnight at 50 °C. Insta-Fluor (5 ml) was added before counting the samples for radioactivity.

RESULTS

Localization of PI-TP in quiescent cells upon stimulation

We studied the localization of PI-TP in exponentially growing, confluent and serum-starved Swiss mouse 3T3 cells. The metabolic activity of confluent and serum-starved cells has strongly decreased as compared with exponentially growing cells [26]. However, in contrast with confluent cells, serum-starved cells still demonstrate some metabolic activity. Therefore we named the serum-starved cells semi-quiescent and the confluent cells quiescent. In agreement with a previous study [18], the amount of PI-TP in exponentially growing 3T3 cells is increased when compared with semi-quiescent cells (Figures 1a and 1c). In both cell cultures the association of PI-TP with the Golgi system is very distinct, as shown by double labelling with TRITC–ricin (Figures 1b and 1d). In addition, in exponentially growing cells, PI-TP is localized in the nucleus and in the cytoplasm (Figure 1a). In order to determine the localization of PI-TP during the transition from quiescence to growth, quiescent 3T3 cells were incubated with PMA and bombesin. Bombesin binds to a specific receptor [23], which activates the phospholipase C-dependent breakdown of PtdInsP_2 [21,22], whereas PMA activates protein kinase C [19]. In quiescent 3T3 cells (7–10 days after reaching confluency) the labelling of PI-TP is diffuse; structural labelling is barely visible (Figure 2a). Upon stimulation of these cells, the labelling pattern rapidly changes. After 10 min of incubation with PMA or bombesin, PI-TP is associated with structures which represent the Golgi system (Figures 2c and 2e). The Golgi structures were identified by double-labelling with TRITC–ricin (results not shown; see Figures 1b and 1d). After 3 h of stimulation, the association of PI-TP with the Golgi system was still observed (Figures 2d and 2f). On the other hand, stimuli which induce cellular processes by mechanisms different from the phosphoinositide cycle (e.g. dexamethasone and dibutyryl cyclic AMP) were found to have no effect on PI-TP localization (results not shown).

These observations strongly suggest that those compounds that activate protein kinase C affect the localization of PI-TP in quiescent 3T3 cells. Attempts to determine the phosphorylation of PI-TP in quiescent cells prelabelled with [^{32}P]P_i have failed because of the very low incorporation of ^{32}P label in proteins under these conditions. For this reason we have investigated the localization of PI-TP in semi-quiescent (serum-starved) cells stimulated by PMA and bombesin (Figure 3). In contrast with quiescent cells, the association of PI-TP with the Golgi system is very distinct (Figures 3a and 3b). In response to PMA, a clear redistribution of PI-TP can be detected within 10 min (Figure

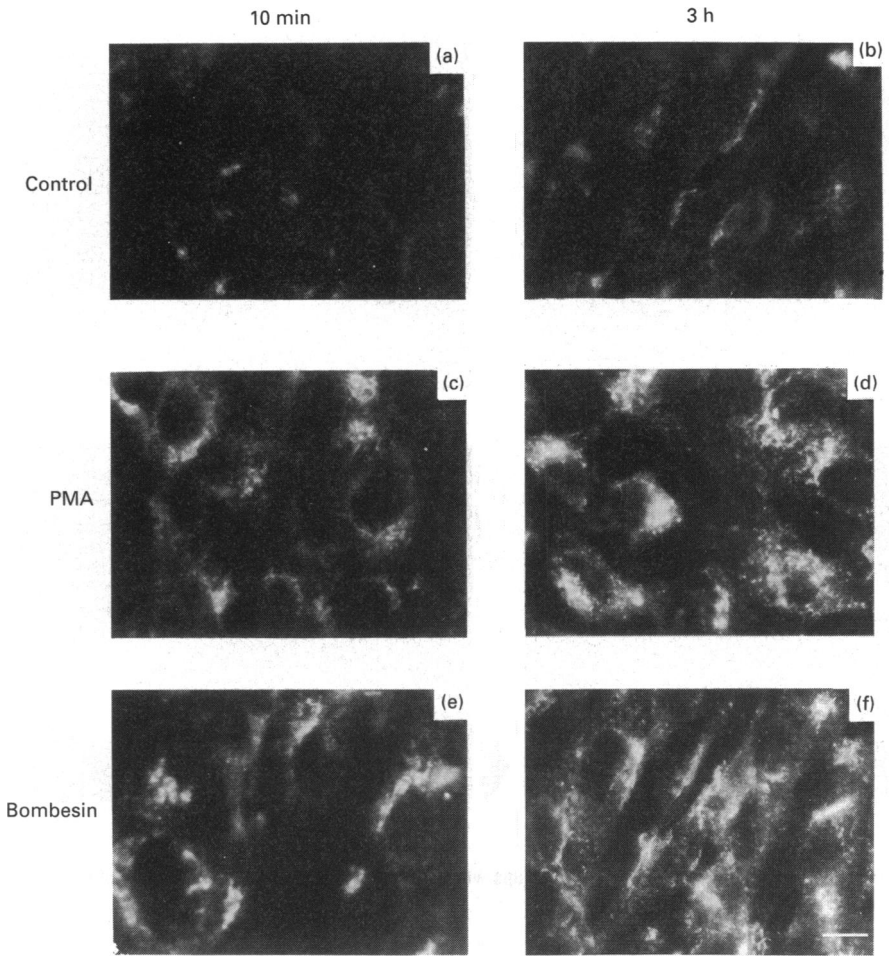

Figure 2 Immunofluorescence localization of PI-TP in quiescent and stimulated Swiss mouse 3T3 cells

Quiescent cells were incubated for 10 min (**a, c, e**) or 3 h (**b, d, f**) with DBH (**a, b**), PMA (50 ng/ml; **c, d**) or bombesin (10 nM; **e, f**). Cells were grown, made quiescent and fixed as described in the Materials and methods section. Bar: 5 μm.

3c). Rounding of the cells was observed after 3 h of PMA treatment (Figure 3d). With bombesin, no change in the localization of PI-TP was apparent after 10 min. However, after 3 h of stimulation, some clear changes in the localization were observed (Figure 3f). Because of the higher metabolic activity of the semi-quiescent cells, it was possible to carry out ^{32}P-labelling studies.

Phosphorylation of PI-TP *in vivo*

Previously we have demonstrated by Western blotting of cytoplasmic, total membrane and nuclear fractions of semi-quiescent and exponentionally growing 3T3 cells that, contrary to what one would expect from the immunofluorescence labelling, PI-TP could only be detected in the cytoplasmic fraction [18]. Similar observations were made with semi-quiescent cells upon stimulation, indicating that, apparently, cell fractionation disrupts the association of PI-TP with the Golgi system. In order to determine whether PI-TP is phosphorylated *in vivo*, it suffices to immunoprecipitate this protein from the cytoplasmic fraction of 3T3 cells. Before immunoprecipitation, semi-quiescent 3T3 cells were incubated for 5 h with [^{32}P]P_i (0.5 mCi/2 ml of medium in a 25 cm^2 dish). Cells were stimulated by incubation with PMA (50 ng/ml) or with bombesin (1 nM) for 10, 30, 60 and 180 min. Phosphorylation of total soluble proteins in control and PMA-stimulated (10 min) cells is shown in Figure 4(a) (lanes 1 and 2 respectively). Before immunoprecipitation of PI-TP, cytoplasmic fractions were pre-cleared by incubation with pre-immune IgG. As shown in Figure 4(a), lanes 3 and 4, the phosphorylated proteins that bind non-specifically to IgG were removed; no PI-TP could be detected. This protein was precipitated by subsequent incubation of the pre-cleared fractions with the affinity-purified anti-PI-TP antibody. As shown in Figure 4(a), lanes 5 and 6, PI-TP is phosphorylated in control cells (lane 5). However, upon stimulation with PMA, the phosphorylation of PI-TP is increased (lane 6). In Figure 4(b) the phosphorylation *in vivo* of PI-TP after stimulation with PMA and bombesin is compared. The increased phosphorylation after 10 min of stimulation with PMA (cf. lanes 1 and 2) is maintained at a comparable level for 30 min, 60 min (results not shown) and 180 min (lane 3). When the cell cultures were stimulated with bombesin, no increase in the phosphorylation of PI-TP could be detected after 10 min (lane 4), 30 or 60 min (results not shown) or 180 min (lane 5). The stoichiometry of the phosphorylation of PI-TP *in vivo* after 10 min of stimulation with PMA has been determined to be 0.4 mol of phosphate/mol of PI-TP. From these studies it can be concluded

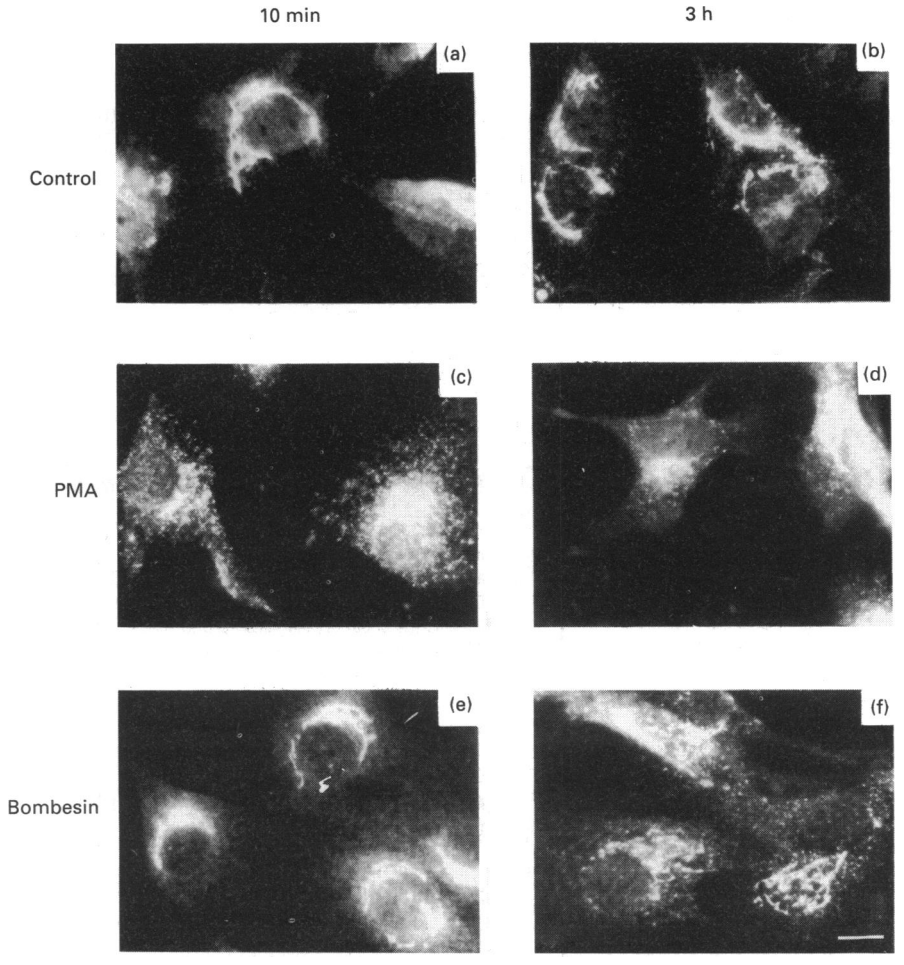

Figure 3 Immunofluorescence localization of PI-TP in semi-quiescent and stimulated Swiss mouse 3T3 mouse cells

Quiescent cells were incubated for 10 min (**a, c, e**) or 3 h (**b, d, f**) with DBH (**a, b**), PMA (50 ng/ml; **c, d**) or bombesin (10 nM; **e, f**). Cells were grown, made semi-quiescent and fixed as described in the Materials and methods section. Bar: 5 μm.

that PI-TP is phosphorylated in 3T3 cells when protein kinase C is activated by PMA. In contrast, despite the effect of bombesin on the intracellular redistribution of PI-TP (Figures 3e and 3f), no apparent increase in phosphorylation of PI-TP was observed upon stimulation with bombesin. In view of the observed effect with PMA, the protein kinase C-dependent phosphorylation of PI-TP was further investigated *in vitro*.

Phosphorylation of PI-TP *in vitro*

In the phosphorylation experiments *in vitro* we have used PI-TP from bovine brain, which we have available in pure form. Since the amino acid sequence of mammalian PI-TP is highly conserved [32], we have assumed that bovine brain PI-TP is comparable with mouse 3T3 fibroblast PI-TP. This is also supported by the high affinity of the anti-brain PI-TP antibody for 3T3 fibroblast PI-TP.

By computer analysis (using the PC Gene program) it was established that rat brain PI-TP [16] contains five serine and threonine residues which could function as potential phosphorylation sites for protein kinase C (Table 1). That indeed PI-TP is a substrate for protein kinase C is shown in Figure 5 (panel a, protein staining; panel b, autoradiogram of ^{32}P-labelled proteins). As shown for two concentrations of PI-TP (Figure 5a, lanes 3–6), this protein is phosphorylated in a Ca^{2+}/phospholipid-dependent way (Figure 5b, lanes 3–6). In incubations without PI-TP, protein kinase C is autophosphorylated; no other phosphorylated bands are visible (Figure 5b, lane 2). This indicates that the enzyme preparation does not contain any other protein that can be phosphorylated in a Ca^{2+}/phospholipid-dependent way.

The phosphorylation of PI-TP by protein kinase C *in vitro* reaches a maximal value after 30 min of incubation; phosphorylation remains stable for at least 1 h (Figure 6a). The autophosphorylation of protein kinase C shows identical kinetics under these conditions (see insert). Phosphorylation as a function of PI-TP concentration is shown in Figure 6(b). From the Lineweaver–Burk plot (see the insert) we have calculated a K_m of 0.85 μM for the phosphorylation of PI-TP (in the presence of 10 μM ATP). The stoichiometry as calculated from incubations with several ATP and PI-TP concentrations is 0.2 mol of phosphate/mol of PI-TP.

We have determined the transfer activity of PI-TP *in vitro* before and after protein kinase C-dependent phosphorylation [28]. No significant effect was observed. This may be due to the low degree of phosphorylation of PI-TP. On the other hand, it

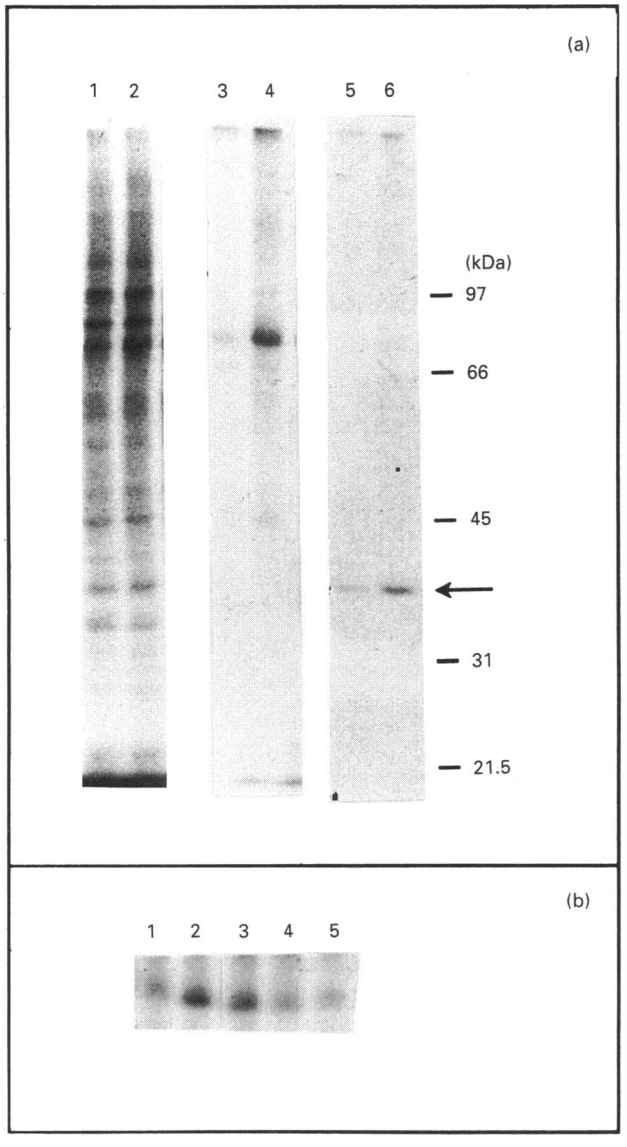

Figure 4 Phosphorylation of PI-TP *in vivo* in Swiss mouse 3T3 cells

Samples were analysed by SDS/PAGE (15% gels) followed by autoradiography. (**a**) Lanes 1 and 2, total ^{32}P-labelled proteins in the cytoplasmic fraction from 3T3 cells; lanes 3 and 4, ^{32}P-labelled proteins cleared from the cytoplasmic fractions by pre-immune IgG; lanes 5 and 6, ^{32}P-labelled PI-TP immunoprecipitated from the pre-cleared cytoplasmic fractions by anti-PI-TP antibody. Lanes 1, 3, 5, control cells; lanes 2, 4, 6, cells incubated for 10 min with PMA (50 ng/ml). Molecular-mass calibration with standard proteins is shown on the right. The arrow indicates PI-TP.(**b**) Immunoprecipitated PI-TP from control cells and from cells stimulated with PMA or bombesin. Lane 1, control cells; lanes 2, 3, cells stimulated with PMA (50 ng/ml) for 10 and 180 min respectively; lanes 4, 5, cells stimulated with bombesin (1 nM) for 10 and 180 min respectively.

Table 1 Protein kinase C-specific phosphorylation sites in the amino acid sequence of rat brain PI-TP

	Amino acid no.	Amino acid sequence		
1	59	EKGGY	T	HKIYH
2	166	PAKFK	S	IKTGR
3	169	FKSIK	T	GRGPL
4	198	AYKLV	T	VKFKW
5	251	RMEEE	T	KRQLD

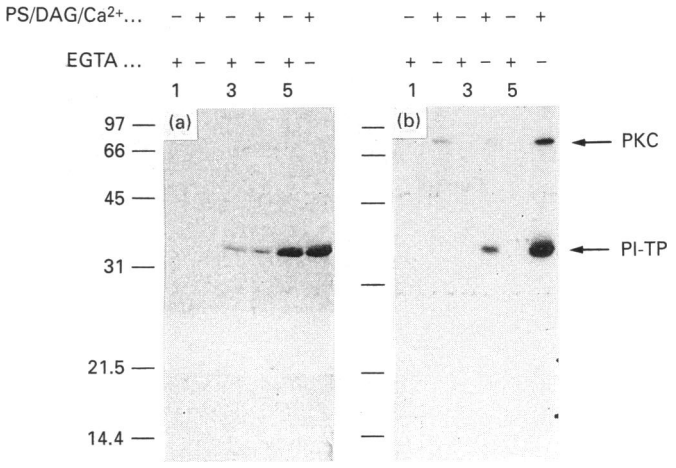

Figure 5 Phosphorylation of PI-TP *in vitro* by protein kinase C

PI-TP (0.3 and 3 μg) and protein kinase C (0.5 μg) were incubated for 20 min as described in the Materials and methods section. The samples were analysed by SDS/PAGE (15% gels) and the gels were stained with Coomassie Brilliant Blue (**a**) or processed for autoradiography (**b**). Phosphorylation was determined in the presence of EGTA (1 mM; lanes 1, 3 and 5) or in the presence of Ca^{2+} (1 mM) and PS/DAG vesicles (96 and 3.2 μg/ml respectively; lanes 2, 4 and 6). Lanes 1, 2, protein kinase C; lanes 3, 4, protein kinase C and 0.3 μg of PI-TP; lanes 5, 6, protein kinase C and 3 μg of PI-TP. The molecular masses (kDa) of standard proteins are indicated on the left-hand side.

remains to be established whether phosphorylation does affect the transfer activity.

DISCUSSION

It was reported recently that PI-TP in yeast is identical with the SEC14 protein involved in secretion, and that this protein is associated with the Golgi system [33]. Recently, we have demonstrated that mammalian PI-TP is also associated with the Golgi system [18]. However, it should be noted that the mammalian and yeast PI-TP lack any significant amino acid sequence similarity [16]. If, similarly to yeast PI-TP, mammalian PI-TP is essential for the functioning of the Golgi system in the secretion process, this would be an example where in evolution two different proteins have functionally converged. In the present study we have investigated the localization of PI-TP in exponentially growing, confluent (quiescent) and serum-starved (semi-quiescent) Swiss mouse 3T3 fibroblast cells. Addition of PMA and bombesin to quiescent cells resulted in a very rapid association of PI-TP with the Golgi system, which was maintained for up to 3 h (Figure 2). In contrast with what is observed in quiescent cells, PI-TP is already substantially associated with the Golgi system in semi-quiescent cells. When these cells are stimulated with PMA, a rapid (within 10 min) redistribution of PI-TP occurred (Figures 3c and 3d). Stimulation of these cells with bombesin also induced redistribution of PI-TP (Figures 3e and 3f). PMA is an activating agent of protein kinase C [19]. Hence it was of great significance to observe that stimulation of the semi-quiescent cells with PMA leads to an increased phosphorylation of PI-TP (Figure 4). Bombesin, a well-known mitogen for 3T3 cells [21–23,25] has been shown to stimulate the hydrolysis of PtdInsP_2 to InsP_3 and DAG in 3T3 cells [22,34]. Furthermore, the involvement of protein kinase C in the cellular response of 3T3 cells to bombesin has also been demonstrated [35,36]. However, when semi-quiescent Swiss 3T3 cells were stimulated with bombesin, we could not detect an increase in the phosphorylation of PI-TP between 10 min and 3 h

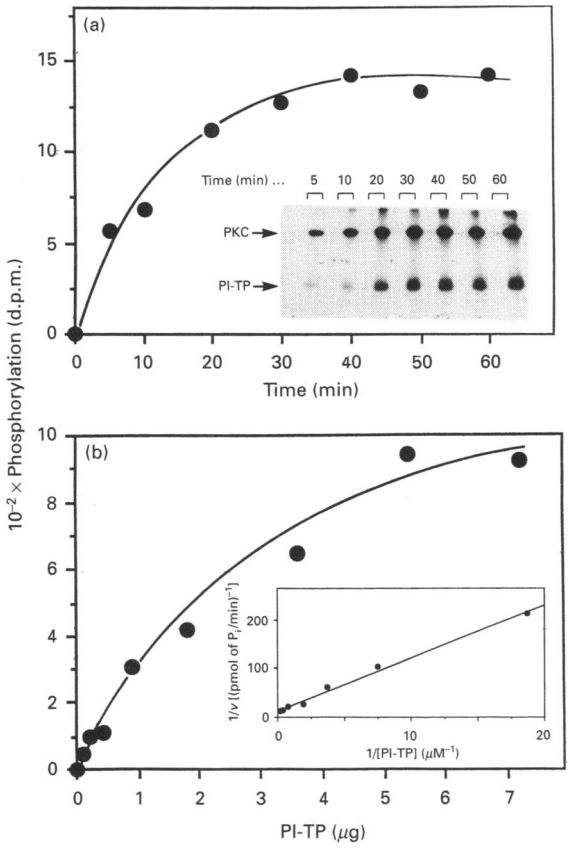

Figure 6 Kinetic analysis of the protein kinase C-dependent phosphorylation of PI-TP

(**a**) Time course. PI-TP (3 μg) and protein kinase C (PKC; 0.5 μg) were incubated as described in the Materials and methods section. After incubation for various periods, the reactions were terminated by addition of trichloroacetic acid. Samples were analysed by SDS/PAGE (15% gels). The insert shows the autoradiograph of the gel. The curve is constructed by counting the radioactivity in the eluents of the excised gel bands. For each time point, the Ca^{2+}/phospholipid-independent and -dependent phosphorylation were determined (first and second lane respectively of each time point). (**b**) Phosphorylation of PI-TP as a function of concentration. Increasing concentrations (0.1–7.2 μg) of PI-TP were incubated with protein kinase C (0.5 μg) for 20 min as described in the Materials and methods section. The Ca^{2+}/phospholipid-dependent and -independent phosphorylation was analysed as described in Figure 4(**a**). The Lineweaver–Burk plot is shown in the insert.

(Figure 4b). Therefore it has to be concluded that activation of protein kinase C does not necessarily lead to an increased phosphorylation of PI-TP. It remains to be established whether this is due to the very transient nature of the activation of protein kinase C after stimulation with bombesin [22,34]. Another explanation for the different effects of PMA and bombesin could be that, in response to bombesin, distinctly fewer proteins are phosphorylated in Swiss 3T3 cells than in response to PMA [37]. Possibly PI-TP belongs to the group of proteins which is not, or barely, phosphorylated upon stimulation with bombesin. In view of recent publications, another explanation for the different effects of PMA and bombesin on the phosphorylation of PI-TP could be that protein kinase C is not responsible for the phosphorylation, but another kinase which is activated by PMA, i.e. MAP kinase (Mitogen Activated Protein kinase, reviewed in [38]). Although the phosphorylation of PI-TP *in vitro* by protein kinase C seems to contradict this possibility, we will investigate whether the phosphorylation of PI-TP is dependent on or mediated by MAP kinase. At this point it is not clear whether, if at all, phosphorylation of PI-TP is a prerequisite for the observed redistribution after stimulation of 3T3 cells with PMA or bombesin. In general, however, protein phosphorylation is known to affect the activity and/or localization of many proteins. For instance, phosphorylation of the eukaryotic initiation factor 4F leads to an increased initiation of protein synthesis [39]. It has been reported for the protein p57 in normal rat kidney cells that its localization at the Golgi system is affected by phosphorylation [40]. An example where phosphorylation affects both localization and activity is presented by CTP:phosphocholine cytidylyltransferase in Chinese hamster ovary cells [41]. The observed phosphorylation of PI-TP after stimulation of cells by PMA suggests that *in situ* PI-TP may be a substrate for protein kinase C. This is supported by the ready phosphorylation of PI-TP by protein kinase C *in vitro*. The affinity of the enzyme for PI-TP, as expressed by the K_m value of 0.85 μM, is comparable with that observed for other protein substrates of protein kinase C [41–43]. Under standard conditions, the stoichiometry of the phosphorylation of PI-TP *in vitro* is 0.2 mol of phosphate/mol of PI-TP. This is less than one would expect from the five theoretical phosphorylation sites predicted to be present per molecule of PI-TP (Table 1). It can be expected that the conditions of phosphorylation *in vitro* are not optimal when PI-TP is used as the substrate. Furthermore, similar values of stoichiometry of phosphorylation have been found for prothrombin (0.2; [44]), phospholipase A_2 (0.5; [45]) and the cellular Kirsten *ras* gene product, p21 (0.6; [46]). The stoichiometry of the phosphorylation of PI-TP *in vivo* is 0.4 mol of phosphate/mol of PI-TP. This relatively low value may be due to the fact that in non-stimulated 3T3 cells PI-TP is already phosphorylated to a certain extent. The presence of both phosphorylated and non-phosphorylated PI-TP in unstimulated Swiss mouse 3T3 cells emphasizes the questions on the possible functions of phosphorylation in (regulation of) localization and activity of mammalian PI-TP. Currently, we are developing methods to purify the phosphorylated PI-TP and the non-phosphorylated PI-TP in order to determine the phosphorylation site and to study the effect of phosphorylation on the properties of PI-TP, e.g. binding and transport of PI and PC and the interaction with membranes.

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