Relationship between phosphoinositide kinase activities and protein tyrosine phosphorylation in plasma membranes from A431 cells

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Production of PtdIns(4)*P* and PtdIns(4,5)*P*₂ by plasma-membrane preparations from A431 cells was selectively stimulated in a dose-dependent manner by epidermal growth factor (EGF) in the presence of Na₃VO₄. Na₃VO₄ itself mimicked this effect, which was overcome after treatment by a specific phosphotyrosyl phosphatase isolated from A431 cells. PtdIns and PtdIns(4)*P* kinase activities were present in phosphotyrosyl-proteins isolated from EGF- and/or Na₃VO₄-stimulated A431 cells by immunoaffinity using an anti-phosphotyrosine antibody. These data suggest for the first time an EGF-dependent regulation of PtdIns 4-kinase and PtdIns(4)*P* 5-kinase activities by a mechanism involving a tyrosine-phosphorylation process.

INTRODUCTION

A431 cells, an epidermoid carcinoid cell line over-expressing EGF receptor, have been much used in studies dealing with the mechanism of signal transduction by growth factors. There is now compelling evidence that, in addition to the stimulation of protein tyrosine phosphorylation (Carpenter, 1987), EGF is able to promote activation of phospholipase C in A431 cells, resulting in the production of two intracellular messengers, Ins(1,4,5)*P*₃ and 1,2-diacyl-*sn*-glycerol, from PtdIns(4,5)*P*₂ (Wahl *et al.*, 1987; Hepler *et al.*, 1987). Besides the activation of phospholipase C, this step could be regulated by the amount of the substrate PtdIns(4,5)*P*₂, which depends on that of its immediate precursor PtdIns(4)*P*.

On the other hand, several reports established a strong relationship between tyrosine kinases and PtdIns kinase (Whitman *et al.*, 1985; Sale *et al.*, 1986; Auger *et al.*, 1989). From recent data, it appears that a lipid kinase introducing phosphate into the 3-position of inositol phospholipids is associated with various protein tyrosine kinases, including oncogene products such as pp60^{src} (Whitman *et al.*, 1987, 1988), the receptor to PDGF (Auger *et al.*, 1989), colony-stimulating factor-1 (Varticovski *et al.*, 1989) and insulin (Endemann *et al.*, 1990). Interestingly, the occurrence of these new phospholipids, namely PtdIns(3)*P* and PtdIns(3,4)*P*₂, has been related to the mitogenic signal generated by PDGF in fibroblasts (Whitman *et al.*, 1987), Balb/c3T3 cells (Kaplan *et al.*, 1987) and smooth-muscle cells (Auger *et al.*, 1989). Furthermore, upon transfection with PDGF-receptor mutants unable to associate with a type I PtdIns kinase (Coughlin *et al.*, 1989), cells were markedly deficient in PDGF-induced mitogenesis, but phosphoinositide hydrolysis by phospholipase C remained intact.

In the present study, we have addressed the question of a possible similar interaction between EGF-receptor tyrosine kinase and PtdIns 3-kinase. Our data reveal that in the plasma membrane of A431 cells EGF-dependent tyrosine phosphorylations were correlated with an increase in the exclusive synthesis of PtdIns(4)*P* and PtdIns(4,5)*P*₂.

MATERIALS AND METHODS

Materials

Percoll [poly(vinylpyrrolidone)-coated silica particles] was from Pharmacia (Uppsala, Sweden). DMEM and fetal-calf serum were from Gibco (Paisley, Renfrewshire, Scotland, U.K.). Calf serum was from I.B.F. (Villeneuve-la-Garenne, France). EGF, PDGF and all other chemicals were obtained from Sigma (St. Louis, MO, U.S.A.). Sepharose-linked monoclonal anti-phosphotyrosine antibody, Hyperfilm-β-max, Na₃³²PO₄, [γ-³²P]ATP (3000 Ci/mmol), [³H]inositol-labelled PtdIns, PtdIns(4)*P* and PtdIns(4,5)*P*₂ were purchased from Amersham International (Amersham, Bucks, U.K.).

Plasma-membrane isolation

Plasma membranes were isolated from cultured A431 cells (about 10⁵ cells/cm²) as previously described (Payraastre *et al.*, 1988), except that lysis buffer contained 50 μg of leupeptin/ml, 2 mM-phenylmethanesulphonyl fluoride and 2 mM-EGTA as protease inhibitors.

Immuno-isolation of phosphotyrosyl-proteins

Cells (about 10⁵ cells/cm²) were washed with 2 × 15 ml of DMEM. Then DMEM containing either Na₃VO₄ (100 μM) or EGF (200 ng/ml) plus Na₃VO₄ (100 μM) was added to the cells for 10 min at 37 °C in a gas phase of 5% (v/v) CO₂ in air. After washing with 2 × 15 ml of DMEM, cells from one plate were incubated for 15 min at 4 °C in 1.5 ml of ice-cold solubilization buffer containing 20 mM-Hepes (pH 7.2), 50 mM-NaCl, 30 mM-Na₄P₂O₇, 5 mM-β-glycerophosphate, 1% Nonidet P-40, 1 mM-EGTA, 1 mM-phenylmethanesulphonyl fluoride, 100 μM-Na₃VO₄ and 10 μg of leupeptin/ml. After centrifugation for 5 min at 12000 *g* (4 °C), the clarified soluble extracts were treated for 2 h 30 min at 4 °C with 50 μl of Sepharose-linked anti-phosphotyrosine antibody. The bead matrix was then batch-washed four times with the following media: (1) 138 mM-NaCl/8 mM-Na₂HPO₄/1 mM-KH₂PO₄ (pH 7.4); (2) 500 mM-LiCl/100 mM-Tris/HCl (pH 7.6); (3) distilled water; (4) 20 mM-

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; PTPase, phosphotyrosine phosphatase.

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Tris/HCl/100 mM-NaCl/1 mM-EDTA (pH 7.6). The phosphoproteins were then eluted from the immunoadsorbent by addition of 100 μ l of buffer A, containing 20 mM-Tris/HCl, 100 mM-NaCl, 10 mM-MgCl₂, 0.5 mM-EGTA, 40 μ M-ATP and 15 mM-phenyl phosphate for 10 min at 4 °C.

Assay of PtdIns and PtdInsP kinase activities

Plasma membranes (about 100 μ g of protein) suspended in 50 mM-Tris/HCl, pH 7.4, were preincubated at 0 °C for 10 min in the presence of 0.07 mM-ATP, 0.5 mM-Na₃VO₄, 10 mM-MgCl₂ and various concentrations of EGF. Assays were started by adding 10 μ Ci of [γ -³²P]ATP and incubated for 10 min at 20 °C, followed by lipid extraction (Bligh & Dyer, 1959; Lloyd *et al.*, 1982) and analysis by t.l.c. (Gonzalez-Sastre & Folch-Pi, 1968) or h.p.l.c., after deacylation performed with methylamine reagent as described by Clarke & Dawson (1981). When incubations were performed with immuno-isolated phosphotyrosyl-proteins, which do not contain endogenous phospholipids, sonicated vesicles of phospholipids were included in the incubation mixture at the following concentrations: PtdIns, 200 μ M; PtdIns(4)P, 50 μ M; PtdIns(4,5)P₂, 10 μ M. Incubations were performed for 10 min at 25 °C.

Phosphotyrosyl phosphatase (PTPase) preparation and assay

The enzyme was isolated from A431 cell extracts, by the procedure of Bütler *et al.* (1989). The final preparation was devoid of any detectable phosphatase activity towards [³²P]phosphoseryl-casein kinase II and [³²P]phosphoseryl- and phosphothreonyl-casein, which were prepared as described previously (Cochet *et al.*, 1983; Cochet & Chambaz, 1983). The enzyme was also inactive towards [³²P]PtdIns(4,5)P₂, prepared as described by Cochet & Chambaz (1986). EGF receptor was ³²P-labelled by an EGF-dependent self-phosphorylation reaction in Triton-X-100-solubilized A431-cell membrane preparations as described by Weber *et al.* (1984), with the difference that the reaction was not stopped by addition of trichloroacetic acid. The ³²P-labelled receptor was then purified through a protamine-Sephadex column-chromatography step, as described by Lokeshwar *et al.* (1989). One unit of PTPase activity was defined as the amount of enzyme preparation releasing 1 fmol of P_i/min from ³²P-labelled EGF receptor.

The phosphatase activity was inhibited by an average of 90 % in the presence of 0.5 mM-Na₃VO₄. These conditions were employed for control experiments using inhibited PTPase (see Fig. 4).

H.p.l.c. analysis of phosphoinositides

Analysis of the deacylated lipids was exactly as described by Auger *et al.* (1989) on the total lipid extract. Authentic tritiated PtdIns, PtdIns(4)P, PtdIns(4,5)P₂ as well as [³²P]phosphatidic acid and [³²P]P_i were used as standards for the identification of the phosphoinositides. Furthermore, the elution profile of the most common inositol phosphates was checked, and these were found to be separated from the deacylated phospholipids. Finally, each assay mixture of deacylated lipids was mixed with a tracer amount of non-labelled ATP and ADP, the A₂₆₀ of which served for standardizing the elution times. These were not significantly different from one run to the other. Radioactivity eluted from the 4.6 mm $\times$ 100 mm Partisphere SAX (Whatman International, Maidstone, Kent, U.K.) column was monitored and quantified by a LB506C detector (Berthold, Munich, Germany), by using the Čerenkov effect for ³²P or after admixture of Liquiscint 303 (Zinsser, Maidenhead, Berks., U.K.) for tritiated samples.

Miscellaneous

Proteins were analysed by SDS/PAGE as described by

Laemmli (1970), in a 10–15 %-acrylamide gradient by using the Phast system (Pharmacia). Proteins were determined as described by Lowry *et al.* (1951).

RESULTS

Effect of EGF and Na₃VO₄ on phosphoinositide phosphorylation in plasma-membrane preparations from A431 cells

Plasma membranes were preincubated at 0 °C in the presence of non-radioactive ATP, EGF and/or Na₃VO₄. Further incubation at 20 °C with [γ -³²P]ATP revealed that incorporation of ³²P into PtdInsP and PtdInsP₂ was stimulated in a dose-dependent manner after exposure to EGF (Fig. 1a). In agreement with the dose-dependence of EGF-receptor self-phosphorylation observed with the same membranes (results not shown), such an optimal dose is in the same range as those observed when the EGF-receptor tyrosine kinase activity is assayed with an exogenous peptide substrate and when the receptor itself is optimally self-phosphorylated (Gill *et al.*, 1984). This effect on phosphoinositide kinases was totally dependent on the presence of Na₃VO₄, known as a specific inhibitor of phosphotyrosyl-protein phosphatases (Swarup *et al.*, 1982). Indeed, a preliminary

Fig. 1. Dose-dependent stimulation by EGF (a) and Na₃VO₄ (b) of ³²P incorporation from [γ -³²P]ATP into PtdInsP and PtdInsP₂.

(a) Plasma membranes from A431 cells (40–60 μ g of protein) were preincubated for 10 min at 0 °C in the presence of 0.07 mM-ATP, 0.5 mM-Na₃VO₄, 10 mM-MgCl₂ and increasing concentrations of EGF. After addition of 10 μ Ci of [γ -³²P]ATP, assays were incubated at 20 °C for 10 min. Polyphosphoinositides were then extracted and analysed as described in the Materials and methods section. Data are expressed as pmol of ³²P incorporated into PtdInsP (●) or PtdInsP₂ (○)/mg of protein and represent means $\pm$ S.E.M. of five separate experiments. (b) Assays were performed as in (a), except that preincubation of plasma membranes was done in the presence of various concentrations of Na₃VO₄, without EGF. Data are expressed as in (a) and are means $\pm$ S.E.M. of four separate experiments.

Fig. 2. Time course of ^{32}P incorporation into PtdInsP: effect of excess unlabelled ATP

Plasma membranes were preincubated in the absence (a) or in the presence (b) of 0.5 mM- Na_3VO_4 ; 10 mM-ATP (unlabelled) was added at the indicated time (arrow).

Fig. 3. H.p.l.c. profiles of the deacylated inositol phospholipids from total lipid extracts

(a) Control plasma membranes and (b) plasma membranes stimulated by 200 ng of EGF/ml as described in the Materials and methods section. The inserts present data on a larger scale. Abbreviation: GroP, glycerophospho.

experiment revealed that stimulation of PtdInsP and PtdInsP₂ phosphorylation by 400 ng of EGF/ml was 5-fold less in the absence of Na₃VO₄. This suggests that inhibition of phosphotyrosyl-protein dephosphorylation was probably necessary to detect an increase *in vitro* in lipid kinase activities induced by EGF. Furthermore, preincubation of plasma membranes with various concentrations of Na₃VO₄ alone also led to an increased labelling of the two phospholipids, although the stimulation was lower than in the presence of EGF (Fig. 1*b*). Since ³²P incorporation might simply reflect an increased turnover of the phosphate moiety present on the inositol ring, pulse-chase experiments were performed to check that point. Upon addition of an excess of unlabelled ATP to the incubation mixture, radioactivity displayed an immediate, very limited, fall, but thereafter remained stable for at least 10 min (Fig. 2*a*), indicating that the activity of PtdInsP phosphomonoesterase remained negligible under our incubation conditions. A very similar picture was observed in the presence of Na₃VO₄ (Fig. 2*b*), excluding the possibility that the stimulatory effect of Na₃VO₄ could be explained by phosphomonoesterase inhibition. Similar results (not shown) were observed for PtdInsP₂.

Characterization of phosphoinositides phosphorylated in plasma membranes from A431 cells upon stimulation by EGF or Na₃VO₄

Fig. 3 shows the h.p.l.c. elution profiles of deacylated lipids extracted from ³²P-labelled plasma membranes stimulated or not by EGF plus Na₃VO₄. The elution pattern was very similar to those observed by others, and allowed the identification of all the detected inositol phospholipids. Comparison of the two elution profiles clearly indicated that, upon treatment with EGF plus Na₃VO₄, plasma membranes displayed increased labelling of PtdIns(4)P and PtdIns(4,5)P₂. On the other hand, it was possible to detect only traces of PtdIns(3,4)P₂ in stimulated plasma membranes. This was not due to technical pitfalls, since in independent experiments PtdIns(3)P and PtdIns(3,4)P₂ were both identified in smooth-muscle cells (results not shown) and human platelets (Sultan *et al.*, 1990) upon treatment with PDGF and thrombin, respectively, in agreement with previously reported data (Auger *et al.*, 1989; Nolan & Lapetina, 1990; Kucera & Rittenhouse, 1990).

Phosphoinositide kinase activities in plasma membrane after treatment with a specific PTPase

In order to check whether protein phosphotyrosyl dephosphorylation could affect the EGF-dependent activation of phosphoinositide phosphorylation, A431-cell plasma membranes were incubated first with the purified PTPase preparation, either in the absence (active PTPase) or in the presence (inhibited PTPase) of Na₃VO₄. EGF-activated ³²P incorporation into PtdIns(4)P and PtdIns(4,5)P₂ was then assayed. As illustrated in Fig. 4, membrane exposure to the PTPase strikingly impaired their ability to synthesize both ³²P-labelled phosphoinositides. Although PtdIns(4)P labelling was inhibited by an average of 60%, that of PtdIns(4,5)P₂ was decreased to about 20%, as compared with control values obtained when the phosphatase was inhibited in the presence of Na₃VO₄. Although these observations did not give direct evidence for tyrosine phosphorylation of the phosphoinositide kinases, they suggest that tyrosine phosphorylation of membrane protein component(s) is involved in the signal-transduction process from EGF-receptor activation to stimulation of phosphoinositide kinase activities.

Fig. 4. Phosphoinositide kinase activities in A431-cell plasma membranes after treatment by a specific PTPase

Assays were performed as in Fig. 1, except that plasma membranes were preincubated for 10 min at 20 °C in the presence of 1.62 units of PTPase, either in the absence (active PTPase, ■) or in the presence (inhibited PTPase, □) of 0.5 mM-Na₃VO₄. EGF, ATP (3 μM), Mn²⁺ (2 mM) and Mg²⁺ (10 mM) were then added, and after 10 min at 20 °C the Na₃VO₄ concentration (0.5 mM) was adjusted in each assay and the reaction was started by adding [γ-³²P]ATP; ³²P incorporation was expressed as pmol/mg of protein, and data are from a typical experiment representative of three experiments with similar results.

Presence of PtdIns 4-kinase and PtdIns(4)P 5-kinase activities in phosphotyrosyl proteins isolated by immunoadsorption from A431 cells

The data in Fig. 5 show that Na₃VO₄ treatment of A431 cells resulted in the immunoadsorption of several phosphotyrosyl proteins containing two major components of 60 kDa and 27 kDa. Although in most experiments the low protein content of the immunoadsorbent eluates could not be determined, Fig. 5(*a*) indicates that the amount of immunopurified proteins was markedly increased after exposure to EGF in the presence of Na₃VO₄. Under those conditions, one would expect self-phosphorylated EGF receptor to be present in the immunisolated membrane proteins. Indeed, an additional 170 kDa protein was detected in this case (Fig. 5*a*) and tentatively identified as the EGF receptor. Interestingly, a good parallelism was found between protein content and phosphoinositide kinase activities in the immunoadsorbent eluates. As illustrated in Fig. 5(*b*), the highest enzymic activity was found in immunoprecipitates obtained from cells treated with EGF plus Na₃VO₄ (11.9 pmol of [³²P]_i incorporated), whereas lipid kinase activities were virtually absent from extracts isolated from control cells (0.2 pmol of [³²P]_i incorporated). H.p.l.c. analysis of the phosphoinositides resulting from the kinase activities present in the immunopurified

Fig. 5. Comparison of protein composition (a) and of lipid kinase activities (b) of anti-phosphotyrosine immunoprecipitates from A431 cells

Phosphotyrosyl proteins immunopurified from A431 cells treated by EGF plus Na_3VO_4 (lanes 1), Na_3VO_4 alone (lanes 2) or without any addition (lanes 3) were submitted to SDS/PAGE (a) or incubated in parallel with phospholipids and $[\gamma\text{-}^{32}\text{P}]$ ATP (b) as described in the Materials and methods section. Numbers in panel (a) indicate the position of molecular-mass standards (kDa). Proteins were detected by silver staining. Panel (b) is an autoradiogram of a thin-layer separation of the reaction products from lipid kinase assays: O, origin.

proteins showed patterns similar to those from plasma membranes, indicating that both PtdIns 4-kinase and PtdIns(4) P 5-kinase activities were present.

DISCUSSION

Four lines of evidence obtained in the present study indicate a close relationship between protein tyrosine phosphorylation and phosphoinositide kinase activities in A431 cells: (i) Na_3VO_4 and EGF plus Na_3VO_4 stimulated the production of both PtdIns P and PtdIns P_2 in isolated plasma membranes; (ii) this effect was overcome by a specific phosphotyrosyl-protein phosphatase; (iii) upon incubation with Na_3VO_4 or EGF plus Na_3VO_4 , proteins isolated from A431 cells by immunoaffinity using an antibody specific for phosphotyrosyl residues displayed significant PtdIns and PtdIns(4) P kinase activities; (iv) using h.p.l.c. separation of deacylated phospholipids, PtdIns(4) P and PtdIns(4,5) P_2 were unambiguously identified as specific products of the stimutable phosphoinositide kinase(s) present in the plasma membrane from A431 cells.

No evidence for a significant stimulation of type I PtdIns kinase phosphorylating the 3-position of inositol ring in phosphoinositides (Whitman *et al.*, 1988) could be obtained under these conditions. This failure could be explained by a cytosolic localization of type I PtdIns 3-kinase and its absence from our membrane preparations. This would imply the enzyme translocation to the plasma membrane, following activation of EGF receptor in intact A431 cells. However, we were unable to identify a PtdIns 3-kinase activity in cytosol from resting A431 cells, using optimal conditions previously defined for the enzyme from 3T3 fibroblasts (Whitman *et al.*, 1987). In the same way, treatment of intact A431 cells by EGF did not reveal any significant increase in the $[\text{H}]$ inositol labelling of PtdIns(3) P and PtdIns(3,4) P_2 (results not shown). This would be in line with previous observations by Pignaturo & Ascoli (1990), who reported that EGF stimulates a net synthesis of PtdIns(3,4) P_2 in MA-10 Leydig tumour cells, but not in A431 cells.

Whether PtdIns(4) P 5-kinase is also regulated by EGF is more difficult to assess from the present data, since a stimulation of PtdIns 4-kinase might be sufficient to explain the increased labelling of both PtdIns(4) P and PtdIns(4,5) P_2 . However, the presence of PtdIns(4) P 5-kinase in proteins immunopurified using an anti-phosphotyrosine antibody would argue in favour of a similar regulation for the two enzymes. This could have an important physiological significance, owing to the role of PtdIns(4,5) P_2 as a source of second messengers.

The fact that both PtdIns 4-kinase and PtdIns(4) P 5-kinase activities are purified together with other proteins bound to an antibody directed towards phosphotyrosyl residues does not imply that the two enzymes are substrates of protein tyrosine kinases. A current concept emerging from the most recent studies is that type I PtdIns 3-kinase associates with phosphotyrosyl proteins such as pp60^{src} (Whitman *et al.*, 1987, 1988) or PDGF receptor (Auger *et al.*, 1989). A similar phenomenon could be suggested here for PtdIns 4-kinase and PtdIns(4) P 5-kinase. Although most of the proteins recovered from anti-phosphotyrosine immunoprecipitates could not be identified by SDS/PAGE, EGF receptor was only detected in proteins isolated from EGF-treated cells. This suggests that other tyrosine kinases and other phosphotyrosyl proteins could be involved in the phosphorylation or in the interaction with PtdIns 4-kinase and PtdIns(4) P 5-kinase in Na_3VO_4 -treated A431 cells. This underlines the need to isolate pure PtdIns 4-kinase and PtdIns(4) P 5-kinase, in order to check whether tyrosine phosphorylation affects directly or indirectly their enzymic activity.

In conclusion, the present data provide the first evidence for a regulation of PtdIns 4-kinase [and possibly PtdIns(4) P 5-kinase] activity by a mechanism involving tyrosine phosphorylation. Furthermore, our results in A431 cells and those of Pignaturo & Ascoli (1990) in MA-10 Leydig cells raise the question about the cellular specificity of the link between EGF receptor and either PtdIns 4-kinase or PtdIns 3-kinase. However, one should notice that EGF is not a mitogen in MA-10 Leydig cells, but modulates the responsiveness of these cells to luteinizing hormone and

human chorionic gonadotropin. Further studies will be necessary to elucidate these differences and delineate the precise role of each species of polyphosphoinositides in various cell functions.

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