Phosphoinositide Kinase, Diacylglycerol Kinase, and Phospholipase C Activities Associated to the Cytoskeleton: Effect of Epidermal Growth Factor

B. Payrastre, P. M. P. van Bergen en Henegouwen, M. Breton,* J. C. den Hartigh, M. Plantavid,* A. J. Verkleij, and J. Boonstra

Department of Molecular Cell Biology, University of Utrecht, 3584 CH Utrecht, The Netherlands; and Unité Institut Nationale de la Santé et de la Recherche Médicale 326, Hôpital Purpan, 31059 Toulouse, France

Abstract. In this paper we demonstrate that cytoskeletons isolated from A431 cells have associated with them high activities of several kinases involved in inositol lipid metabolism, such as phosphatidylinositol kinase, phosphatidylinositol phosphate kinase, and diacylglycerol kinase. In addition also phospholipase C activity was detected on isolated cytoskeletons. Controlled extraction of the cytoskeletons followed by in vitro polymerization of actin demonstrated an association of the kinases to the actin filament system consisting of actin and a number of actin-binding proteins. The cytoskeleton-associated lipid kinase activities were significantly increased upon treatment of intact cells with EGF. These data suggest that the association of the phosphoinositide kinases, diacylglycerol kinase, phospholipase C, and also the EGF receptor to the cytoskeleton may play a role in the efficient signal transduction induced by EGF, by providing a matrix for the various components involved in signal transduction.

In this respect it is of interest to note that evidence is accumulating that the growth factor receptor population is at least partly associated to the cytoskeleton. A structural association of the EGF receptors to the cytoskeleton of A431 cells was demonstrated using a variety of electron microscopical methods, while biochemical analysis showed that this fraction is strongly enriched in high affinity receptors (21, 23, 32, 34, 38). The high affinity class of receptors is primarily responsible for EGF-induced signal transduction (12), suggesting strongly that the association of the receptors to the cytoskeleton has an important function in EGF-induced signal transduction. An attractive hypothesis in this respect appears to be a coordinating function of the cytoskeleton, in that the cytoskeleton provides the matrix to which various components of the signal transduction cascade, such as the phosphoinositide kinases, are associated, thus enabling a highly efficient system for activation of one enzyme by the other. According to this hypothesis it is proposed that a number of these enzymes are associated to the cytoskeleton and affected by the activation of the EGF receptor. Therefore we have investigated the interactions between the cytoskeleton and a number of components involved in the phosphoinositol metabolism, since these enzymes, i.e., PtdIns-kinase and PtdInsP-kinase were found previously to be activated by binding of EGF to its receptor (28, 35).

Here we demonstrate that cytoskeletons isolated from different cell lines all contain PtdIns-kinase, PtdInsP-kinase, and DAG-kinase activities. The activities were found in the actin fraction of the cytoskeleton. In addition, it is shown that EGF binding to intact cells causes an increase in the ac-

---

1. Abbreviations used in this paper: DAG, 1,2 diacylglycerol; IP₂, inositol 1,4,5 trisphosphate; KI, potassium iodine; PLC, phospholipase C; PS, phosphatidylserine; PtdIns, phosphatidylinositol; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdInsP₂, phosphatidylinositol bisphosphate; PtdIns(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; PtdOH, phosphatidic acid.
tivity of cytoskeleton-associated kinases. The effect of EGF requires an intact receptor kinase, as deduced from studies using a kinase inactive receptor mutant. Identification of the products generated by the kinases using HPLC analysis demonstrated mainly the presence of PtdIns 4-kinase and PtdIns(4)P 5-kinase while some PtdIns 3-kinase activity was detectable. In addition, evidence is provided that PLC activity is also associated with the cytoskeleton of A431 and other cells.

Materials and Methods

Cell Culture

Mouse NIH 3T3 fibroblast cell lines transfected with human EGF receptor cDNA were kindly provided by Dr. J. Schlessinger (Department of Pharmacology, New York University Medical Center, New York). NEF cells contain wild type EGF receptor, K721A cells express a tyrosine kinase deficient EGF-receptor due to the substitution of Lys 721 by Ala 721 (19). These cells, as well as A431 and Rat1 cells were cultured in DME supplemented with 7.5% FCS (Integro, Zaandam, The Netherlands) and buffered with NaHCO3 in a 7% CO2-humidified atmosphere.

Cytoskeleton Extraction

Cells were grown to 105 cells/cm2 in 10-cm-diam culture dishes (Costar, Cambridge, MA), whether or not stimulated with 200 ng/ml EGF during 10 min at 37°C in DME. Cytoskeletons were prepared essentially as described (34), briefly by extraction with 0.5% Triton X-100, in 20 mM Hepes (pH 7.4), 50 mM NaCl, 1 mM EGTA, 1 mM PMSF, 10 µg/ml leupeptin, and 100 µM sodium orthovanadate for 20 min at 4°C under gentle shaking and then centrifuged for 20 min at 4°C at 40,000 g. The supernatant was dialyzed in a big volume (1 liter) of buffer containing 10 mM Pipes (pH 6.8), 1 mM EGTA, 2 mM MgCl2, 1 mM PMSF, 5 mM benzamidine, 100 µM sodium orthovanadate at 4°C for 3 h. Actin was then polymerized and this suspension was centrifuged at 12,000 g for 5 min at 4°C, the pellet was resuspended in buffer A for 20 min at 4°C under gentle shaking and dialyzed against the same buffer once more. Supernatants and pellets (F-actin-rich fraction) from both polymerization–depolymerization cycles were used for lipid kinase assays.

Preparation of the Polymerized Actin and Actin-binding Protein–rich Fraction

The cytoskeletons from subconfluent A431 cells (15 dishes of 10-cm diam) were solubilized in 10 ml of buffer A containing 0.6 M KI, 100 mM Pipes (pH 6.5), 100 mM KCl, 10 µg/ml leupeptin, 1 mM PMSF, and 100 µM sodium ortho-vanadate for 20 min at 4°C. The supernatant was dialyzed in a big volume (1 liter) of buffer containing 10 mM Pipes (pH 6.8), 1 mM EGTA, 2 mM MgCl2, 1 mM PMSF, 5 mM benzamidine, 100 µM sodium ortho-vanadate at 4°C for 3 h. Total cellular protein (34) was solubilized in electrophoresis sample buffer, boiling for 5 min and separated on 10% polyacrylamide gels and blotted onto nitrocellulose (Schleicher and Schuell, Dassel, Germany). The nitrocellulose was blocked with 0.3% milkpowder in PBS for 45 min at 37°C. The filters were incubated with the different antibodies for 60 min diluted in 0.03% milkpowder in PBS at room temperature. After washing in the same buffer the filter was incubated with secondary antibodies conjugated with alkaline phosphatase. The antibody reaction was visualized in PBS for 45 min at 37°C. The filters were incubated with the different antibodies for 60 min diluted in 0.03% milkpowder in PBS at room temperature. After washing in the same buffer the filter was incubated with secondary antibodies conjugated with alkaline phosphatase. The antibody reaction was visualized in 100 mM Tris-HCl (pH 9.5) 100 mM NaCl, 50 mM MgCl2 containing 0.33 mg/ml nitroblue tetrazolium salt (Sigma Chemical Co.) and 0.17 mg/ml 5-bromo-4-chloro-3-indolylphosphate toluidinium salt (Sigma Chemical Co.).

Results

Phosphoinositide and Diacylglycerol Kinase Activities Associated to the Cytoskeleton of A431 Cells

Treatment of A431 cells with 0.5% Triton X-100 for 15 min at 4°C resulted in extraction of ~85% of the lipids and ~65% of total cellular protein (34). As shown in Fig. 1, the remaining structures contained no recognizable membrane structures, indicating the validity of the Triton X-100 extraction to isolate cytoskeletons. Under these conditions the cytoskeletons contained an intact actin filament system consisting of actin and a large number of actin-associated proteins, the intermediate filament system (see also Fig. 3, lanes 2), as well as the nucleus. As shown in Fig. 2 (lane J), incubation of isolated cytoskeletons of A431 cells with PtdIns and [γ-32P]ATP resulted in the formation of PtdInsP and PtdInsP2, demonstrating the presence of PtdIns-kinase and PtdInsP-kinase in isolated cytoskeletons. Similarly, incubation of cytoskeletons with PtdIns(4)P or 1,2-diolein in the presence of [γ-32P]ATP resulted in the formation of PtdInsP (lane 2) and phosphatidic acid (PtdOH) (lane 3), respectively. Thus in addition to PtdIns-kinase, isolated cytoskeletons contain also PtdInsP-kinase and DAG-kinase activities. In the absence of NH4OH (90/70/20) as a solvent (15). Radioactive lipids were detected by autoradiography, scraped off and the radioactivity was quantified.

When the HPLC technique was used, lipids were decolourized from the total lipid extract as described (1). Authentic tritiated PtdIns, PtdIns(4)P, PtdIns(4,5)P2, as well as [32P]Phosphatidic acid and [32P]Phosphatidate were used as standards for the identification of the phosphoinositides. Radioactivity eluting from the 4.6 mm × 100 mm Partisphere SAX (Whatman International, Maidstone, UK) column was monitored and quantified by a Berthold LB506C detector (Munich, Germany), using the Cerenkov effect for 32P or after addition of Liquestic 303 (Zinsser, Maidenhead, UK) for triturated samples.

Lipid Analysis

Inositol lipids and phosphatidic acid were separated by thin-layer chromatography on silicagel-coated plates using chloroform/methanol/4:3 M ortho-vanadate at 4°C resulted in extraction of almost all the lipids and ~65% of total cellular protein (34). As shown in Fig. 1, the remaining structures contained no recognizable membrane structures, indicating the validity of the Triton X-100 extraction to isolate cytoskeletons. Under these conditions the cytoskeletons contained an intact actin filament system consisting of actin and a large number of actin-associated proteins, the intermediate filament system (see also Fig. 3, lanes 2), as well as the nucleus.
exogenous lipids hardly any activity of the kinases was detected (see lane 3 for PtdIns-kinase and PtdInsP-kinase and lanes 1 and 2 for DAG-kinase), demonstrating that the cytoskeletons are virtually devoid of endogenous phosphoinositides and DAG. The formation of both PtdInsP and PtdInsP$_2$ (lane 1), when the cytoskeletons are incubated in the presence of PtdIns, indicates the presence of a highly active PtdInsP-kinase which phosphorylates PtdInsP immediately following its formation by PtdIns-kinase.

**Association of Phosphoinositide Kinases and DAG-Kinase Activities with the Actin Filament System**

As shown above, phosphoinositide kinase and DAG-kinase activities were detected in the cytoskeleton of A431 cells. The cytoskeleton of these cells is composed of three different filament systems: actin filaments, microtubules, and intermediate filaments (Fig. 3, lanes 1) and in addition these isolated cytoskeletons contained also the nucleus of the cells (Fig. 1). To discriminate between kinase activities in the different cytoskeletal components, the cytoskeletons were, as in the previous experiment, isolated at 4°C which induced the removal of tubulin (Fig. 3, lanes 2), furthermore by solubilization with potassium iodide (KI), which is known to cause a rapid depolymerization of actin filaments, the intermediate filaments consisting of keratin were separated from the actin filaments (Fig. 3, lanes 3) (17). All lipid kinases were active in this KI-treated suspension, demonstrating that KI treatment has no effect on the kinase activities.

After centrifugation, as indicated in Material and Methods, the pellet contained the nuclear fraction as well as the intermediate filaments (not shown), while the supernatant was strongly enriched in actin and actin-binding proteins (Fig. 3, lanes 3).

Subsequent dialysis of the supernatant resulted in a complexation of actin probably because of actin polymerization. Centrifugation of this fraction yielded a supernatant that contained only some PtdInsP-kinase activity (Fig. 4, lane 1) and a pellet containing PtdIns-kinase, PtdInsP-kinase, and DAG-kinase activities (Fig. 4, lane 2). The polymerized actin was subsequently treated with KI and dialyzed again as described above for a second cycle of polymerization–depolymerization. Again, all kinase activities were detected in the polymerized actin fraction (Fig. 4, lane 4), while the supernatant fraction contained only traces of kinase activities (Fig. 4, lane 3). Similar results were obtained in the presence of colchicine as precaution to prevent a possible tubulin polymerization in case some traces of tubulin would still be present in the preparation and not detected by immunoblotting.

Despite the fact that we could measure some activities in the pellet containing the nuclear fraction and the intermediate filaments (not shown), these data demonstrate clearly that the activities of PtdIns-kinase, PtdInsP-kinase, and DAG-kinases are partly associated with the actin filament system (see also Fig. 3, lanes 3), suggesting that the kinases are associated to actin or to actin-binding proteins.
Effect of EGF on Cytoskeleton-associated Lipid Kinase Activities

Recently it has been demonstrated that PtdIns 4-kinase and PtdIns (4)P 5-kinase could be activated by binding of EGF to its receptor, suggesting a close structural association between the EGF receptor and the kinases (9, 28, 35) as has been shown for PLC-γ and the EGF receptor (25). Since it was demonstrated recently that the high-affinity class of EGF receptors is structurally and functionally associated to the cytoskeleton (21, 23, 32, 34, 38), we have studied the effect of EGF on cytoskeleton-associated phosphoinoside kinases. As shown in Fig. 5, addition of EGF (200 ng/ml) to intact cells, followed by the isolation of cytoskeletons and incubation of the cytoskeleton with [γ-32P]ATP and exogenous lipids as described under Materials and Methods, resulted in an increase of the activities of all kinases measured. The effects of EGF on kinase activity may be due to either an in-
Figure 4. Formation of phosphorylated lipids by lipid kinases present in the F-actin-rich fraction. Exogenous lipid vesicles of final concentration of 50 μM PtdIns, PtdIns(4)/P, 1,2-diolein, and 100 μM PS were added, in the presence of [γ-32P]ATP, to the supernatant of the first repolymerization (A, lane 1), the pellet of the first repolymerization (F-actin-rich fraction) (A, lane 2), the supernatant of the second polymerization (B, lane 3), and the pellet of the second polymerization (F-actin-rich fraction) (B, lane 4), respectively. The amount of protein is adjusted to 30 μg in each assay. The phosphorylated lipids were localized by autoradiography, the position of mentioned standards after migration on TLC are indicated, while the arrow shows the origin.

crease in the number of enzyme copies present in cytoskeletons from EGF-treated cells or a changed activity of a single kinase molecule. In this respect it is important to note that after exposure of the cells to EGF, the cytoskeletons contained approximately a 6% increase in protein content. Thus the increase of kinase activity in cytoskeletons of EGF-treated cells occurred simultaneously with an increase in protein content of the cytoskeletons. To discriminate between a direct effect of EGF on the kinases and an indirect effect due to an EGF-induced association of the kinases to the cytoskeleton, the enzyme kinetics were studied in more detail.

The substrate dependency of the kinases was determined in cytoskeletons isolated from EGF-treated and -untreated A431 cells. As shown in Fig. 6A, the PtdIns-kinase activity increased at increasing concentrations of PtdIns, until a maximum activity was reached at around 100–200 μM PtdIns in cytoskeletons of EGF treated as well as untreated cells. Analysis of these data according the Michaelis–Menten saturation kinetics revealed that EGF caused an increase of the Vmax from 4.7 to 6.3 pmol/min/mg protein, while the Km was slightly decreased from 43 to 31 μM. Similar results were obtained for PtdInsP-kinase as shown in Fig. 6B. Anal-

Figure 5. Effect of EGF treatment on the cytoskeleton-associated enzyme activities. Autoradiography of a TLC showing the formation of phosphorylated lipids by cytoskeletons from resting (1) and EGF stimulated A431 cells (2). Lipid kinase assays were performed as described in Fig. 2, protein concentrations were adjusted to 20 μg.

Eogenous substrate concentrations were 200 nM and the protein concentrations determined from control or EGF-treated A431, NEF, and K721A cells. The exogenous substrate concentrations were 200 uM and the protein concentrations 15 ± 4 nM. Results are expressed as percentage of control and are means ± SEM of four to six different experiments.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>PtdIns-kinase</th>
<th>PtdInsP-kinase (percent of control)</th>
<th>DAG-kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>A431</td>
<td>133 ± 16</td>
<td>171 ± 6</td>
<td>205 ± 35</td>
</tr>
<tr>
<td>NEF</td>
<td>124 ± 4</td>
<td>163 ± 14</td>
<td>180 ± 38</td>
</tr>
<tr>
<td>K721A</td>
<td>85 ± 16</td>
<td>109 ± 9</td>
<td>108 ± 4</td>
</tr>
</tbody>
</table>

Lipid kinases were assayed as described in Material and Methods in cytoskeletons from control or EGF-treated A431, NEF, and K721A cells. The exogenous substrate concentrations were 200 uM and the protein concentrations 15 ± 4 nM. Results are expressed as percentage of control and are means ± SEM of four to six different experiments.

Table I. Effect of EGF on Lipid Kinase Activities in Different Cell Lines in the Presence of an Excess of Substrate

Identification of the Phosphoinositide Kinases

Since two types of PtdIns-kinases responsible for the production of various species of polyphosphoinositides have been described (36, 37), we next identified the phosphoinositides formed in isolated cytoskeletons of A431 cells by HPLC analysis of deacylated 32P-lipids. As shown in Fig. 7 A, when PtdIns was added to the cytoskeleton from EGF-treated cells, the main phosphoinoside formed was identified as PtdIns(4)P. In addition also a significant amount of PtdIns(4,5)P was identified, indicating the presence of both PtdIns-4-kinase and PtdIns(4)P 5-kinase in isolated cytoskeletons. Furthermore, the presence of traces of PtdIns(3)P indicated a weak type 1 PtdIns-kinase activity which is in our conditions negligible in comparison with the type 2 PtdIns-kinase. Same species were observed in cytoskeletons isolated from control cells (data not shown) indicating that EGF treatment does not induce the synthesis of new compounds. Addition of PtdIns(4)P to the cytoskeletons resulted in the formation of PtdIns(4,5)P, and PtdOH, these observations demonstrate the presence of PtdIns(4,5) 5-kinase, and DAG-kinase in the A431 cytoskeletons (Fig. 7 B). However, an interesting observation regards the formation of PtdOH, because this indicates that the formation of PtdIns(4,5)P is probably followed by hydrolysis to DAG, the substrate of DAG-kinase. Since we have shown above that isolated cytoskeletons contain only traces of endogenous DAG (Fig. 2 and Fig. 6 C), the formation of PtdOH was only possible if the cytoskeletons contained PLC activity or by accumulation of DAG upon activation. To clarify these possibilities in more detail, cytoskeletons of A431, Rat1, and NEF cells were isolated and subsequently incubated with [3H]PtdIns(4,5)P. As shown in Table II, under these conditions all cytoskeletons were able to form significant amounts of inositol trisphosphate, demonstrating the presence of an active PLC, apparently associated with the cytoskeletons.
(4)P 5'-kinase, DAG-kinase, and PLC activities are associated with the cytoskeletons of a number of different cell lines, including A431, NEF, and Rat1 cells. As such these data are in agreement with the findings that the phosphoinositide kinases are associated to the membrane skeleton of platelets and erythrocytes (11, 26). In addition also traces of PtdIns(3)P were found in cytoskeletons of A431 cells, indicating the presence of a PtdIns 3-kinase even if under our experimental conditions its activity is much lower than the PtdIns 4-kinase. PtdIns 3-kinase has been shown to be able to interact with EGF receptor only in certain cells (4, 9), however its mechanism of regulation by EGF remains unclear (8). Using selective extraction protocols, we provide evidence that the kinases are associated to the actin filamental system of the cytoskeleton, in addition to the nucleus as reported in the literature (10).

These findings are interesting in light of the fact that also a significant fraction of the EGF receptor population has been shown to be associated to the cytoskeleton (38). Further characterization of this receptor class revealed that the receptors display high affinity for EGF (34), i.e., the class of receptors which has been shown to be primarily responsible for EGF induced signal transduction (12). In this paper, we demonstrate in addition that EGF binding to the receptor on intact cells results in an increase of the activities of the kinases on the cytoskeleton. The EGF effect appeared to be dependent on an active receptor kinase since EGF treatment of K721A cells expressing a tyrosine kinase deficient EGF receptor has no effect on these activities. At this moment it is difficult to discriminate between a direct stimulation of the kinases by the EGF receptor and an increase of kinase activity caused by the EGF-induced increase of protein content of the cytoskeletons. The kinetic analysis of kinase activity suggests that the increase should be due to the latter interpretation. A similar phenomenon has been shown for EGF receptor itself (34) and since some lipid kinase activities were found to be associated to the EGF receptor (9), this may explain in part the increase in lipid kinase activities in cytoskeletons from EGF-treated cells. On the other hand, preliminary results have shown that EGF may have also a direct effect on kinase activity since a significant increase in lipid kinase activities was measured after addition of EGF directly to isolated cytoskeletons.

Of particular interest is the observation that incubation of isolated cytoskeletons in the presence of PtdInsP results in the formation of PtdInsP2 and PtdOH. The formation of PtdOH suggests the presence of PLC activity on cytoskeletons, in addition to DAG-kinase, as demonstrated in Table II. Altogether the data presented show that PtdInsP, PtdInsP2, and DAG-kinase are partly associated to the actin filament system. It has been suggested recently that components of the phosphoinositide cycle may be closely related to the regulation of cytoskeletal structures. Thus PtdInsP2 has been demonstrated to bind specifically to profilin, resulting in a dissociation of the profilin–actin complex and consequently in enhanced actin polymerization (22). A similar phenomenon may occur upon EGF treatment allowing an increase in the association of actin-binding proteins to actin, possibly including the kinases measured in this study, as well as the EGF receptor itself. Such a mechanism could explain the formation of so-called signal particles, in which the EGF receptor associates with its targets in a par-

### Table II. PLC Assay against Exogenous Substrate in Cytoskeleton from Resting Cells

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Specific activity (pmol x min^{-1} x mg^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>A431</td>
<td>7.4 ± 2.4</td>
</tr>
<tr>
<td>NEF</td>
<td>6.3 ± 3.3</td>
</tr>
<tr>
<td>K721A</td>
<td>4.0 ± 1.3</td>
</tr>
<tr>
<td>Rat1</td>
<td>4.9 ± 2.3</td>
</tr>
</tbody>
</table>

Cytoskeleton-associated phospholipase C was determined using [3H]PtdIns (4,5)P2 as exogenous substrate. Results are expressed in picomoles of inositol trisphosphate produced per minute and mg of protein (pmol x min^{-1} x mg^{-1}) and are means ± SEM from four to eight different experiments. When BSA (30 μg) alone was added as control, 1 ± 0.3 pmol x min^{-1} x mg^{-1} were detected.
ticular domain at the cell surface. In view of the results presented here and previously, the actin filament system may provide the structural basis for this signal particle formation. Further studies are now in progress to study the role of the actin filament system in more detail.

The authors would like to thank Dr. W. Berendes for help with the scanning electron microscopy and Dr. J. Schlessinger for kindly providing the NEF and K721A cells.

This work was supported by a grant from the Commission of European Communities and Association pour la Recherche sur le Cancer (France).

Received for publication 22 February 1991 and in revised form 14 May 1991.

References


