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

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*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.

THE hydrolysis of a minor membrane phospholipid, phosphatidylinositol 4,5 bisphosphate (PtdIns[4,5]$P_2$),[1] by a specific phospholipase (PLC) is one of the earliest key events in growth factor–induced signal transduction (7, 30). Activation of PLC results in the hydrolysis of PtdIns-(4,5)$P_2$ and the subsequent formation of the second messengers inositol 1,4,5 trisphosphate ($IP_3$) and diacylglycerol (DAG) (18). The $IP_3$ is responsible for the liberation of $Ca^{2+}$ from intracellular organelles, while DAG is involved in the activation of protein kinase C (2). This signal transduction cascade, known as the phosphoinositide pathway, is activated by a wide variety of growth factors, including EGF (16, 20, 27, 29) and PDGF (3). In addition to the role of phosphoinositides in signal transduction, they have been demonstrated to be involved in the regulation of the organization of the cytoskeleton (22, 39). As shown by Lassing and Lindberg (22) PtdIns(4,5)$P_2$ interacts specifically with profilin thereby efficiently dissociating the profilin–actin complex. This dissociation in its turn will yield an increased actin polymerization. Recently it has been demonstrated that binding of PtdIns(4,5)$P_2$ to profilin may play an important role in the hydrolysis of PtdIns$P_2$ (14) and the regulation of PLC$\gamma$1 by profilin and tyrosine phosphorylation by the EGF receptor (13).

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 phosphoinositide 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; $IP_3$, inositol 1,4,5 trisphosphate; KI, potassium iodine; PLC, phospholipase C; PS, phosphatidylserine; PtdIns, phosphatidylinositol; PtdIns(4)P, phosphatidylinositol 4 phosphate; PtdIns$P_2$, phosphatidylinositol biphosphate; PtdIns(4,5)$P_2$, phosphatidylinositol 4,5 biphosphate; 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 Ratl cells were cultured in DME supplemented with 7.5% FCS (Integro, Zaandam, The Netherlands) and buffered with $NaHCO_3$, in a 7% $CO_2$-humidified atmosphere.

### Cytoskeleton Extraction

Cells were grown to $10^5$ cells/cm$^2$ 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. After 15 min at 4°C the cytoskeletons were scraped off with a rubber policeman and pelleted in eppendorff tubes at 12,000 *g* for 1 min (4°C). The cytoskeletons from each dish were washed twice at 4°C in 1 ml of the same buffer but without Triton X-100 and finally resuspended in 50 mM Tris/HCl (pH 7.2) and immediately used for enzymatic assay. All chemicals used were obtained from Sigma Chemical Co. (St. Louis, MO).

### Scanning Electron Microscopy of A431 Cytoskeletons

A431 cells were grown on glass dishes (1-cm diam) and the cytoskeletons were prepared exactly as above but not scraped off. After washing in situ on the glass, the cytoskeletons were fixed in 0.5% glutaraldehyde and 2% paraformaldehyde in PBS for 25 min and dried using increasing concentrations of ethanol. Scanning EM was performed as previously described (6).

### Lipid Kinase Assays

Since no or little endogenous lipids are present in the cytoskeleton, activities were measured in 100 μl containing 50 mM Tris/HCl (pH 7.4), 10 mM $MgCl_2$, 60 μM cold ATP, indicated concentrations of exogenous lipids, and cytoskeleton proteins. Phosphatidylinositol (PtdIns) phosphatidylinositol 4 phosphate (PtdIns [4] P, and DAG were independently mixed or mixed together when indicated with double amounts of phosphatidylserine (PS) dried under $N_2$ stream, and vesicles were made by adding 50 mM Tris/HCl and sonication. The reaction was started by adding 10 μCi [$\gamma$-$^{32}$P]ATP (3,000 Ci/mmol; Amersham International, Amersham, UK) and carried out at room temperature under shaking for 10 min. After stopping the reaction by addition of 400 μl of a mixture of chloroform/methanol (vol/vol), the lipids were immediately extracted (5, 24).

### Assay of Phospholipase C against Exogenous Substrate

Cytoskeleton-associated phospholipase C activity was determined using [$^3$H]PtdIns(4,5)$P_2$ (Amersham International, Amersham, UK) as exogenous substrate. [$^3$H]phosphatidylinositol (PtdIns$P_2$) (30,000 dpm, 1 nmol/assay) were dried under nitrogen stream, and dissolved in 5% sodium cholate, the reaction was performed as described (31).

### Lipid Analysis

Inositol lipids and phosphatidic acid were separated by thin-layer chromatography on silicagel-coated plates using chloroform/methanol/4.3 M $NH_4OH$ (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 deacylated from the total lipid extract as described (1). Authentic tritiated PtdIns, PtdIns(4)P, PtdIns(4,5)$P_2$, as well as [$^{32}$P]phosphatidic acid and [$^{32}$P]orthophosphate 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 $^{32}$P or after admixture of Liquiscint 303 (Zinsser, Maidenhead, UK) for tritiated samples.

### 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 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 $MgCl_2$, 1 mM PMSF, 5 mM benzamidine, 100 μM sodium ortho-vanadate 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 again under the same conditions. Supernatants and pellets (F-actin-rich fraction) from both polymerization-depolymerization cycles were used for lipid kinase assays.

### SDS-PAGE and Immunoblotting

Protein determination was done using BCA-reagent (Pierce Chemical Company, Rockford, IL) with BSA as standard. Proteins were 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 100 mM Tris-HCl (pH 9.5) 100 mM NaCl, 50 mM $MgCl_2$ 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 *1*), incubation of isolated cytoskeletons of A431 cells with PtdIns and [$\gamma$-$^{32}$P]ATP resulted in the formation of PtdInsP and PtdIns$P_2$, 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 [$\gamma$-$^{32}$P]ATP resulted in the formation of PtdIns$P_2$ (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

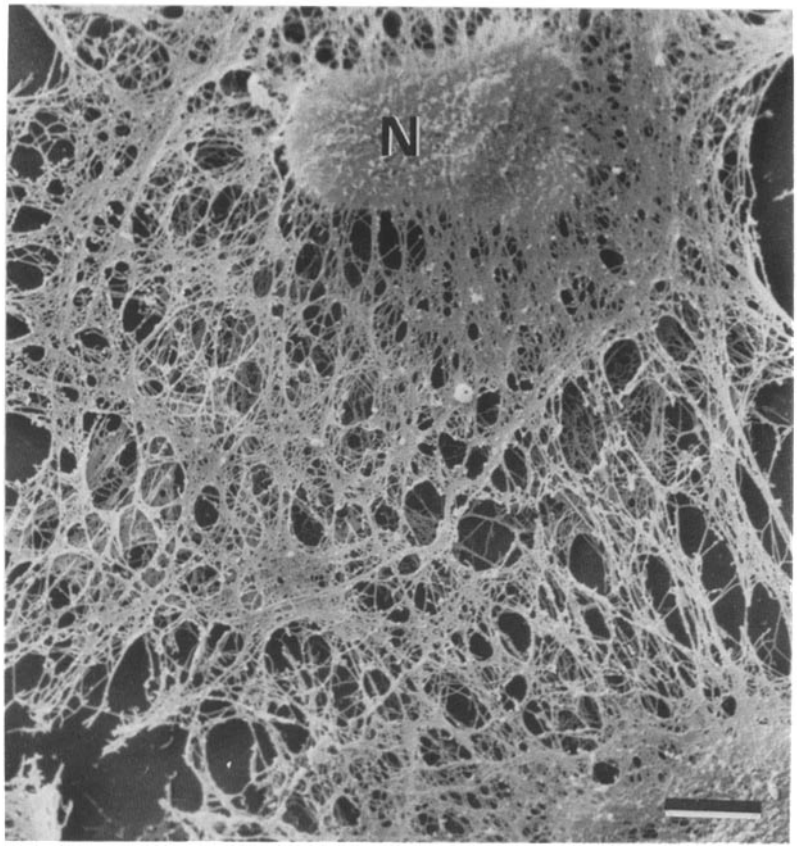



*Figure 1.* Scanning EM of A431 cytoskeleton. A431 cytoskeletons were isolated, fixed, and dried as indicated in Materials and Methods. *N*, nucleus. Bar, 3 $\mu$m.

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 iodine (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.

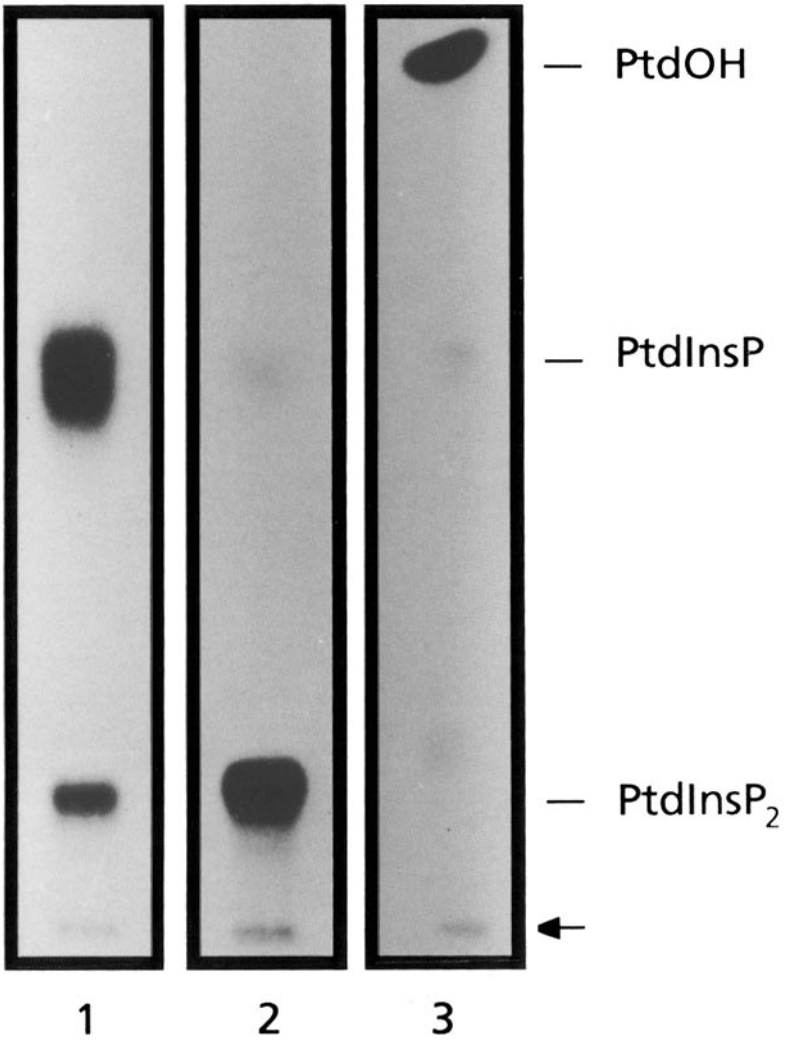



*Figure 2.* Formation of phosphorylated lipids in A431 cytoskeletons. Cytoskeletons (20 μg proteins) from resting A431 cells were incubated with [γ$^{32}$P]-ATP and exogenous lipid vesicles containing, respectively, as final concentration: PtdIns (50 μM)/PS (100 μM; lane *1*), PtdIns(4)P (50 μM)/PS (100 μM; lane *2*), and 1,2-diolein (50 μM)/PS (100 μM; lane *3*). The radioactive lipids were then separated by thin layer chromatography and localized by autoradiography. The positions of mentioned standards after migration are indicated, while the arrow shows the origin.

### *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-$^{32}$P]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-

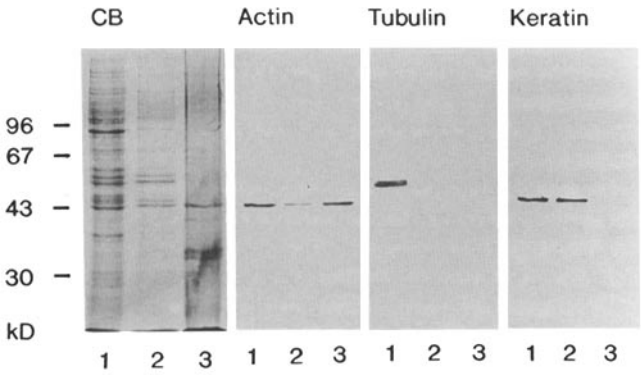



*Figure 3.* Detection of cytoskeletal proteins. A431 cells were extracted with 0.5% Triton X-100 for 15 min at 4°C as indicated in Materials and Methods. The resulting cytoskeletons were further extracted with 0.6 M KI in buffer A for 20 min at 4°C and centrifuged at 4,000 *g*. The supernatant was dialyzed against 10 mM Pipes (pH 6.8), 1 mM EGTA, 2 mM $MgCl_2$, 1 mM PMSF, 5 mM benzamidine, and 100 mM sodium ortho-vanadate at 4°C which promotes actin polymerization. After two subsequent polymerization and depolymerization cycles the actin filaments (F-actin–rich fraction) were pelleted. Protein from 2.5 × $10^4$ intact A431 cells (*1*) and cytoskeletons (*2*) together with 1.25 μg isolated actin-containing filaments (*3*) were separated on 10% SDS-PAGE (*CB*, Coomassie blue stained gel; $10^5$ cells/cytoskeletons, 5 μg actin filaments) and analyzed for cytoskeletal proteins as actin, tubulin and keratin 18 as described in Materials and Methods.

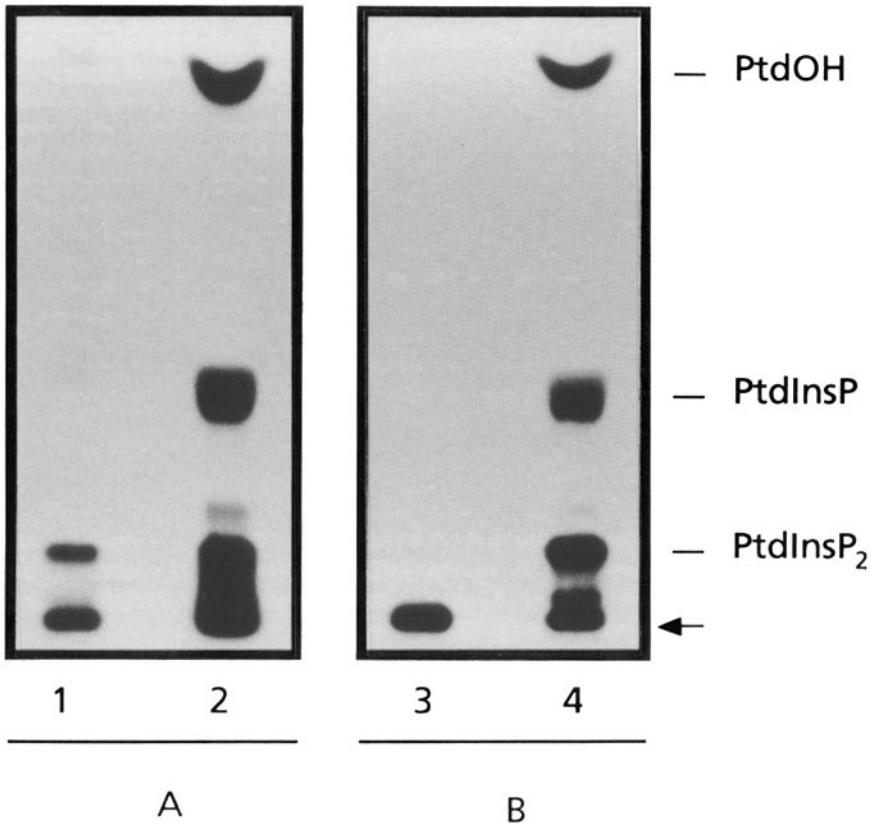
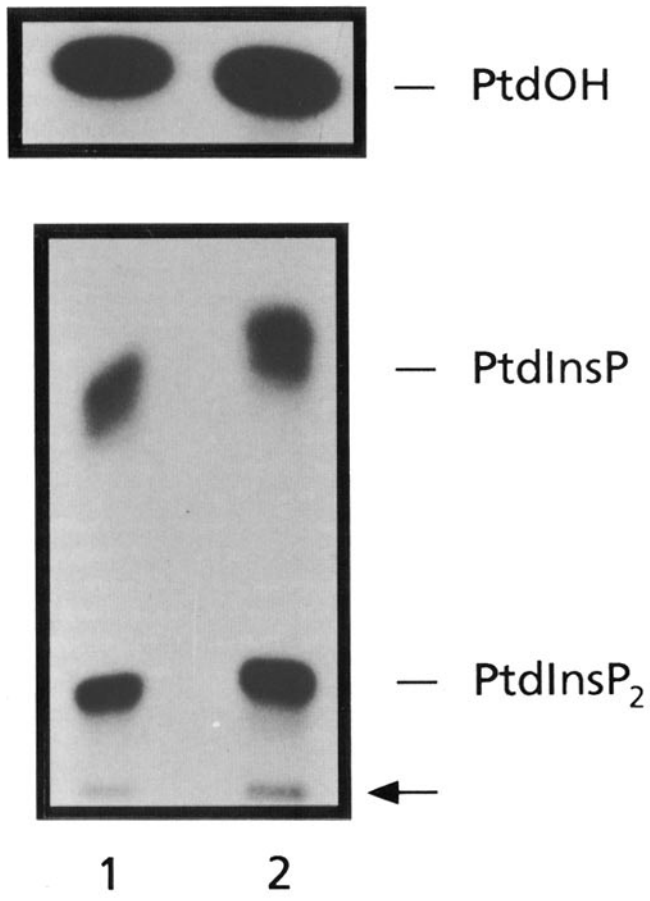



*Figure 4.* Formation of phosphorylated lipids by lipid kinases present in the F-actin-rich fraction. Exogenous lipid vesicles of final concentration of 50 $\mu$M PtdIns, PtdIns(4)/P, 1,2-diolein, and 100 $\mu$M PS were added, in the presence of [$\gamma^{32}$P]ATP, to the supernatant of the first repolymerization (*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 $\mu$g 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. 6 *A*, the PtdIns-kinase activity increased at increasing concentrations of PtdIns, until a maximum activity was reached at around 100–200 $\mu$M 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 $V_{max}$ from 4.7 to 6.3 pmol/min/mg protein, while the $K_m$ was slightly decreased from 43 to 31 $\mu$M. Similar results were obtained for PtdInsP-kinase as shown in Fig. 6 *B*. 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 $\mu$g.

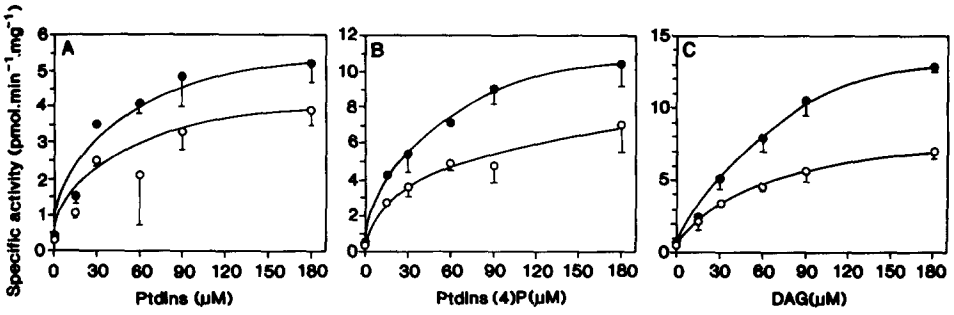



*Figure 6.* Substrate dependency of the different lipid kinases. Reactions were performed as described in Material and Methods. Results are represented as mean ± SEM from three to four different experiments. (*A*) Substrate dependency of PtdIns-kinase associated with cytoskeleton from control A431 cells (○) or EGF treated A431 cells (●). Protein concentrations were adjusted in each assay to 25 μg. (*B*) Substrate dependency of PtdInsP-kinase associated with cytoskeleton from control A431 cells (○) or EGF treated A431 cells (●). Protein concentrations were adjusted in each assay to 10 μg. (*C*) Substrate dependency of DAG-kinase associated with cytoskeleton from control A431 cells (○) or EGF treated cells (●). Protein concentrations were adjusted in each assay to 15 μg.

ysis of these data demonstrated that EGF caused an increase of $V_{max}$ from 7.7 to 12.3 pmol/min/mg protein, while in this case the $K_m$ appeared to be unchanged at 35–36 μM. The strongest effect of EGF was, however, observed for DAG-kinase (Fig. 6 *C*). EGF caused an increase of $V_{max}$ from 8.9 to 19.1 pmol/min/mg protein, while the $K_m$ increased from 52.8 to 83.3 μM. These results demonstrate that the $K_m$'s are not changed markedly, but the $V_{max}$ is clearly increased, suggesting that the effect of EGF is primarily due to an increased association of the kinases to the cytoskeleton, as was reported previously for the EGF receptor (34). On the other hand preliminary results indicate that EGF is able to activate the lipid kinases directly on isolated cytoskeletons from A431 cells (not shown). After addition of 200 ng/ml EGF to isolated cytoskeletons an almost twofold increase in phosphoinositide kinase and DAG-kinase activities was observed, indicating that EGF may also have a direct effect on the kinase activities.

To demonstrate that the effect of EGF on phosphoinositide kinase activities is a specific receptor-mediated effect, the lipid kinase activities were studied in cytoskeletons isolated from K721A cells. This cell line is transfected with a mutant EGF receptor in which lysine 721 was replaced by alanine, yielding a kinase deficient receptor (19). As a control we used NEF cells, a NIH-3T3 cell line transfected with a wild type EGF receptor (19, 33). The lipid kinases were determined in cytoskeletons from EGF-treated and -untreated cells, in the presence of excess of substrate. As shown in Table I, EGF increased these activities in cytoskeletons of NEF cells, but not in K721A cells. These data clearly demonstrate that the EGF receptor protein tyrosine kinase is essential for the increase of cytoskeleton-associated phosphoinositide kinase and DAG-kinase activities.

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

| Cell lines | PtdIns-kinase | PtdInsP-kinase (percent of control) | DAG-kinase |
|---|---|---|---|
| A431 | 133 ± 16 | 171 ± 6 | 205 ± 35 |
| NEF | 124 ± 4 | 163 ± 14 | 180 ± 38 |
| K721A | 85 ± 16 | 109 ± 9 | 108 ± 4 |

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 μM and the protein concentrations 15 ± 4 μg. Results are expressed as percentage of control and are means ± SEM of four to six different experiments.

### *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 $^{32}$P-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)$P_2$ 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)$P_2$ and PtdOH, these observations demonstrate the presence of PtdIns(4)P 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)$P_2$ 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, Ratl, and NEF cells were isolated and subsequently incubated with [$^3$H]-PtdIns(4,5)$P_2$. 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.

## *Discussion*

In this paper we demonstrate that PtdIns 4-kinase, PtdIns-

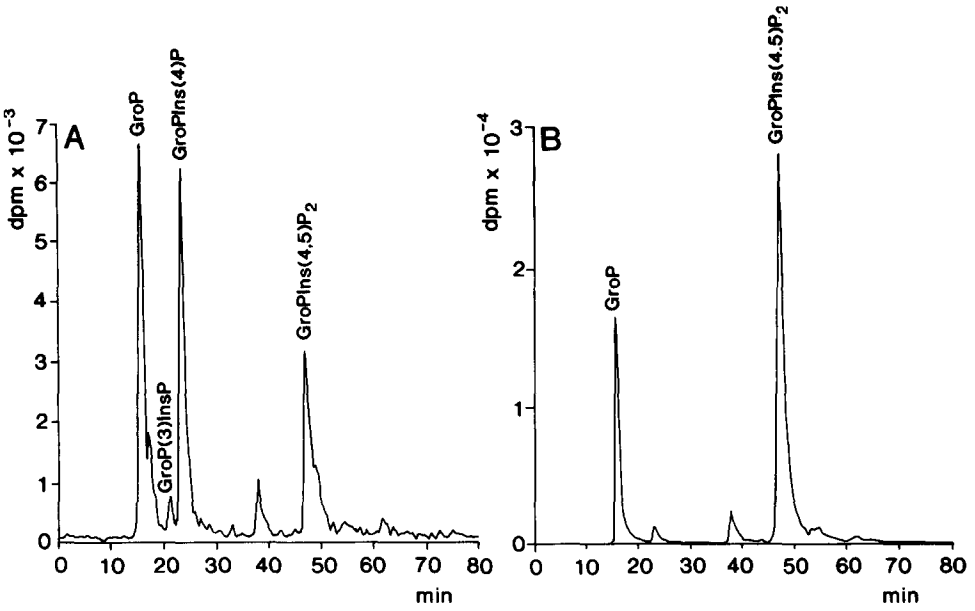



*Figure 7.* HPLC profiles of deacylated phospholipids obtained from lipid kinase activities measured in cytoskeletons from EGF stimulated A431 cells. Cytoskeletons (40 μg protein) from EGF stimulated A431 cells were incubated 10 min at 32°C with [γ-$^{32}$P]ATP and exogenous lipid vesicles containing, respectively, as final concentration PtdIns (200 μM)/PS (400 μM) (*A*) and PtdIns-(4)P (200 μM)/PS (400 μM) (*B*). Lipids were extracted, deacylated, and separated on HPLC column as described in Material and Methods. *GroP*, glycerophospho.

(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 Ratl 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. PdtIns 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.

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

| Cell lines | Specific activity |
|---|---|
| A431 | 7.4 ± 2.4 |
| NEF | 6.3 ± 3.3 |
| K721A | 4.0 ± 1.3 |
| Ratl | 4.9 ± 2.3 |

Cytoskeleton-associated phospholipase C was determined using [$^3$H]PtdIns (4,5)$P_2$ as exogenous substrate. Results are expressed in picomoles of inositol trisphosphate produced per minute and mg of protein (pmol × min$^{-1}$ × 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 × min$^{-1}$ × mg$^{-1}$ were detected.

Of particular interest is the observation that incubation of isolated cytoskeletons in the presence of PtdInsP results in the formation of $PtdInsP_2$ 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 PtdIns-kinase, PtdInsP-kinase, 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 $PtdInsP_2$ 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-

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.

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