Epidermal growth factor induces serine phosphorylation of actin

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Abstract Stimulation of cells by epidermal growth factor induces a rapid polymerisation of actin in the cortical skeleton. Activation of the EGF-receptor leads to autophosphorylation and to phosphorylation of specific intracellular substrates. Here we show that actin is phosphorylated in vitro and in vivo upon EGF stimulation. Two-dimensional phospho-amino acid analysis shows that phosphorylation occurs on serine, not on tyrosine residues.

Key words: EGF; EGF-receptor; Actin; Phosphorylation

1. Introduction

The cytoskeleton, and especially the actin microfilament system, plays an important role in growth factor-induced signal transduction. For example, EGF-receptor kinase activation leads to the phosphorylation of various cytoskeleton associated proteins [1–6]. Key enzymes in the EGF signal transduction cascade, like phosphatidylinositol kinase, phosphatidylinositol-4-phosphate kinase, diacylglycerol kinase and phospholipase C are associated with the cytoskeleton [7]. In addition it has been demonstrated that stimulation of cells with EGF or other ligands affects actin organisation. Using immunofluorescence microscopy and confocal laser scanning microscopy it has been shown that EGF not only causes changes in the actin distribution but also induces a significant increase in actin polymerisation at the apical membrane-skeleton [8,9]. Furthermore it has been shown that immunoprecipitation of the cytoskeleton-associated EGF-receptor resulted in co-immunoprecipitation of actin [8], while selective extraction of cytoskeletons revealed an interaction between EGF-receptor and actin filaments [10]. Using highly purified components it has been demonstrated that the EGF-receptor can bind directly to actin via its cytoplasmic domain with no other proteins involved [11]. Other growth factor receptors such as NGF-receptor [12,13] and PDGF-receptor [14] are also associated with the cytoskeleton.

As described above, a number of actin binding proteins, like fodrin, spectrin, tubulin and ezrin, have been demonstrated to become phosphorylated upon activation of cells by growth factors [1–6]. Of particular interest, however, is the observation that actin itself may be phosphorylated. Previously it has been shown that PKC can phosphorylate actin, phosphorylating G-actin more efficiently than F-actin, but these studies were carried out exclusively in vitro. This phosphorylated actin was found to be more efficiently incorporated into F-actin than unphosphorylated actin. In contrast, phosphorylation of actin by PKA was found to polymerise less well than the phosphorylated form [15]. The *Physarum polycephalum* actin–fragmin complex is phosphorylated on threonine residues of actin by a monomeric 80 kDa protein and casein kinase I. The actin phosphorylation–dephosphorylation mechanism is suggested to regulate the organisation of the microfilament system in this organism [16]. In *Dictyostelium* cells tyrosine phosphorylation of actin was found to increase when oxidative phosphorylation was inhibited by 2,4-dinitrophenol [17]. It has been suggested that in these cells the phosphorylation of actin influences the equilibrium between G- and F-actin or that the phosphorylation of actin influences its association with other proteins. Starved *Dictyostelium* cells that return to growth medium show an increase in the amount of phosphorylated actin in time. Howard and coworkers have shown that changes in tyrosine phosphorylation of actin correlates with changes in cell shape of *Dictyostelium* cells and therefore it may play a role in actin polymerisation–depolymerisation [18]. Tyrosine phosphorylation of actin has also been found in an in vitro kinase assay with partially purified insulin receptor [19].

Since we have recently demonstrated that the EGF-receptor is an actin binding protein, it was of interest to establish whether actin itself is phosphorylated upon activation of EGF-receptor tyrosine kinase activity. We demonstrate that indeed activation of the EGF-receptor tyrosine kinase results in the phosphorylation of actin in vivo and in vitro. However, the actin appears to be phosphorylated on serine residues rather than on tyrosine residues, demonstrating that actin itself is not a direct substrate of the EGF-receptor, but is a target of downstream kinase activities.

2. Materials and methods

2.1. Tissue culture

HER14 (NIH3T3 cells transfected with EGF-receptor cDNA, a gift from Dr. J. Schlessinger) fibroblasts and A431 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, UK) supplemented with 7.5% w/v fetal calf serum (FCS) (Gibco, Paisley, UK).

2.2. Membrane preparations

Plasma membranes were prepared according to the method described by Thom et al. [20] with minor changes. Instead of using large amount of cells, the preparations were performed from 5–10 162-cm^2 dishes. No filtration through nylon gauze was performed. The plasma-membrane rich pellet was resuspended in phosphate buffered saline (PBS) with 20% glycerol and when required stored at −20°C.

2.3. ^{32}P-labeling of intact HER14 cells

HER14 cells were grown on 75-cm^2 dishes, serum starved (DMEM,

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Abbreviations: ATP, adenosine triphosphate; DMEM, Dulbecco, modified Eagle's medium; EDTA, ethylene diamide tetra acetic acid; EGF, epidermal growth factor; FCS, fetal calf serum; NGF, nerve growth factor; PDGF, platelet-derived growth factor; PKA, protein kinase A; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulphate; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electroforesis; TCA, trichloroacetic acid; TLC, thin layer chromatography.

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0% FCS) overnight and incubated in 5 ml phosphate-free DMEM containing 4 mCi [$^{32}P_i$]orthophosphate (Amersham International, UK) at 37°C for 4 h. Subsequently, cells were stimulated with EGF (50 ng/ml) for 10 min and actin was immunoprecipitated from the cells.

2.4. Immunoprecipitation of actin and EGF-receptor

Actin and EGF-receptor were immunoprecipitated from whole cell lysates or membrane preparations. Cells or membrane preparations were lysed in RIPA buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% Triton, 0.1% SDS, 1 mM EDTA, 1 mM PMSF, 1 mM Benzamidine, 100 mM NaF, 1 mM Na_3VO_4) at 4°C for 10 min and centrifuged for 5 min at 12,000 × *g* in an Eppendorf centrifuge. The supernatants were incubated with a 1 : 1 suspension of protein-A-Sepharose for 1 h at 4°C and centrifuged. The supernatants were either incubated with 1 μg of anti-actin antibody (ICN Biomedicals, USA) or with 1 μg of anti-EGF-receptor antibody (Santa Cruz Biotechnology, Santa Cruz) for 3 h at 4°C. Subsequently protein-A-Sepharose was added and after a further incubation overnight the immunoprecipitates were washed three times, once with RIPA buffer, once with 0.5 M NaCl, 20 mM Tris-HCl pH 7.4, 1% Triton, 1 mM PMFS, 1 mM Benzamidine, 1mM Na_3VO_4, and finally with 0.15 M NaCl, 20 mM Tris-HCl pH 7.4, 1% Triton, 1 mM PMFS, 1 mM Benzamidine, 1 mM Na_3VO_4. The beads were boiled in 20 μl Laemmli sample buffer for 5 min and samples were separated by 10% SDS-PAGE.

2.5. Phosphoamino acid analysis

Phosphorylated actin bands were excised from stained and dried gels, allowed to swell in 500 μl 0.05 M ammonium bicarbonate for 5 min. The gel piece was homogenised and another 500 μl 0.05 M ammonium bicarbonate was added together with 50 μl β-mercaptoethanol and 10 μl 10% SDS. Samples were boiled for 10 min and rotated overnight at 37°C. Protein was precipitated with TCA and washed with 100% ethanol. Pellet was dissolved in 50 μl 6 N HCl and hydrolysed at 110°C for 1 h, the pellet was resuspended in water and lyophilised overnight. Samples were resuspended in buffer pH 1.9 (2.2% formic acid, 7.8% acetic acid) containing phosphoserine, phosphothreonine and phosphotyrosine as markers. The samples were spotted on a TLC plate and subjected to two-dimensional high voltage thin layer chromatography. The first dimension was run at 1.5 kV with buffer pH 1.9. The second dimension was run at 1.3 kV with buffer pH 3.5 (5% glacial acetic acid, 0.5% pyridine). Markers were visualised by ninhydrin and radioactivity by autoradiography.

2.6. Kinase assay

Membranes (30 μg) were incubated for 10 min at room temperature with 100 ng EGF in a volume of 30 μl. The reaction was started by adding 10 μl 1.5 M ammonium sulphate and 20 μl reaction buffer (60 mM HEPES pH 7.4, 15 mM $MgCl_2$, 6 mM $MnCl_2$, 60 mM Na_3VO_4, and either 10 μCi [γ-^{32}P]ATP (Amersham International, UK) or 60 μM ATP (Boehringer Mannheim, Mannheim). The mixture was incubated for 20 min at 37°C, after which an immunoprecipitation of actin was performed. For assays carried out in the presence of protein kinase inhibitors, EGF was added together with inhibitors at concentrations varying from 10–500 μM.

3. Results

3.1. In vitro phosphorylation of actin

It was demonstrated previously that the EGF-receptor is an actin binding protein [11] and since actin can be phosphorylated in vitro by several protein kinases [15–19], we have studied the possibility that actin is phosphorylated by the EGF-receptor tyrosine kinase. Therefore in vitro kinase assays were performed using membranes isolated from Her14 and A431 cells as described in section 2. EGF was added to the membranes for 10 minutes at room temperature and a kinase assay was performed as described in section 2. Actin was then immunoprecipitated from the membranes and samples were separated on a 10% SDS gel, which was subsequently subjected to autoradiography. As shown in Fig. 1A, addition of EGF to membranes isolated from A431 cells resulted in an increase in the phosphorylation state of the EGF-receptor (lane 1 without EGF, lane 2 with EGF). A similar increase was observed in the phosphorylation state of the EGF-receptor upon addition of EGF to membranes isolated from HER14 cells (compare lane 3 and 4). Fig. 1B shows phosphorylation of actin immunoprecipitated from the same membranes as the EGF-receptor. Again a clear phosphorylation of actin after EGF stimulation is detected (lane 2 and 4) in both cell types as compared to the unstimulated membranes (lanes 1 and 3). These data demonstrate clearly that in vitro actin becomes phosphorylated upon EGF stimulation. No actin was added to these membranes, indicating that the actin phosphorylated in this manner is part of the membrane skeleton.

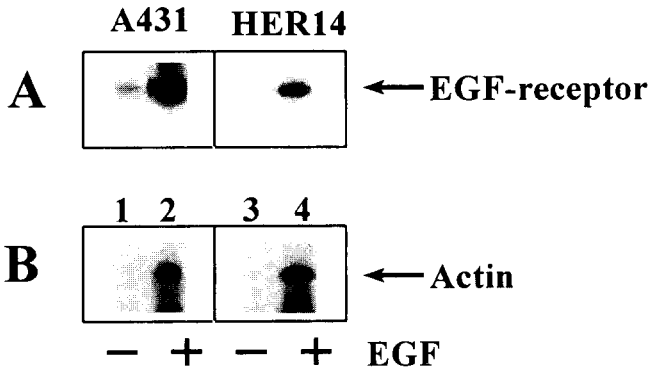

Fig. 1. EGF stimulation of actin phosphorylation in vitro. Thom membranes prepared from A431 or HER14 cells were either left untreated (lanes 1 and 3), or stimulated with 200 ng EGF (lanes 2 and 4) for 10 min at room temperature before performing an in vitro kinase assay. Actin and the EGF-receptor were immunoprecipitated as described in section 2, and separated by 10% SDS-PAGE. Arrows indicate bands corresponding to phosphorylated EGF-receptor (A) and actin (B).

Since the EGF-receptor is an actin binding protein it is tempting to speculate that actin is a direct substrate of the EGF-receptor. To establish the role of the EGF-receptor tyrosine kinase in more detail, a kinase assay was performed in the presence of tyrphostin-47, a tyrosine kinase inhibitor specific

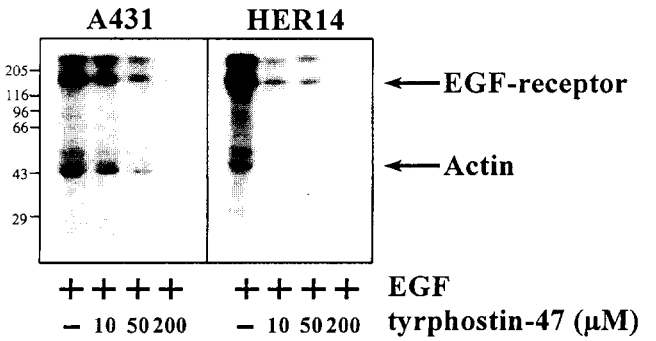

Fig. 2. Actin phosphorylation in vitro is inhibited by the tyrosine kinase inhibitor tyrphostin-47. Thom membranes prepared from A431 or HER14 cells were stimulated with 200 ng of EGF and increasing concentrations of tyrphostin-47 (10, 50 and 200 μM) for 10 min at room temperature before performing an in vitro kinase assay. Actin was immunoprecipitated which resulted in a co-immunoprecipitation of the EGF-receptor and the immunoprecipitates were seperated by 10% SDS-PAGE. Arrows indicate bands corresponding to phosphorylated EGF-receptor and actin.

for the EGF-receptor [21]. Membranes were treated with EGF and increasing concentrations of tyrphostin-47. Subsequently actin was immunoprecipitated as described in section 2. In Fig. 2 both actin and the EGF-receptor are visible which is a result of a co-immunoprecipitation of the EGF-receptor with actin. As shown in Fig. 2 both the phosphorylation of the EGF-receptor and actin decreases by increasing concentrations of tyrphostin-47 for both cell types. The decline in phosphorylation in HER14 cells is more rapid than in A431 cells, which contain more EGF-receptors. These data indicate that actin phosphorylation induced by EGF in vitro is directed by the EGF-receptor tyrosine kinase activity. Subsequently a Western blot was prepared of kinase assay samples prepared with cold ATP. This Western blot was incubated with anti-phospho-tyrosine antibody (UBI, Lake Placid USA). Fig. 3 shows the bands representing the EGF-receptor in A431 and HER14 cells upon EGF addition but no bands representing actin was detected. These findings demonstrate that phosphorylation of actin upon EGF stimulation is, in fact, not on tyrosine residues, and therefore in vitro actin appears not to be a substrate of the EGF-receptor itself, but from another kinase whose activity is regulated by EGF through the EGF-receptor.

3.2. *In vivo phosphorylation of actin*

Phosphorylation of actin in vivo was studied in HER14 cells. HER14 cells were labelled with [^{32}P]orthophosphate for 4 h, before stimulation with 50 ng/ml EGF for 10 min. After immunoprecipitation of actin, samples were separated on a 10% SDS gel (Fig. 4). Fig. 4A shows a band of phosphorylated actin in the stimulated cells (lane 2) but not in the control cells (lane 1). The remaining three bands are seen in both the control cells and the stimulated cells. These are probably phosphoproteins binding to actin. Figure 4B shows the phosphorylation of the EGF receptor in stimulated (lane 4) and unstimulated (lane 3) cells, immunoprecipitated from the same cell lysates as a control for the EGF receptor activity. In vivo actin becomes phosphorylated by stimulation of cells with 50 ng/ml EGF for 10 min. To determine which amino acid residue is

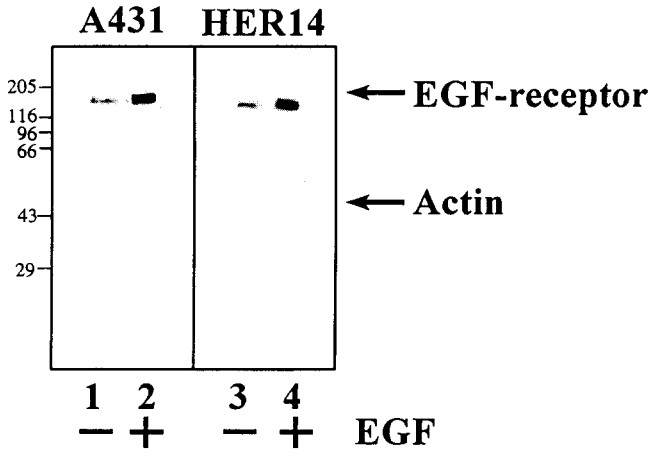

Fig. 3. Anti-phosphotyrosine Western analysis of EGF stimulated actin phosphorylation. Thom membranes prepared from A431 or HER14 cells were used to phosphorylate actin in vitro as described above. Actin immunoprecipitates were separated by 10% SDS-PAGE, and transferred to nitrocellulose. Blots were incubated with anti-phosphotyrosine antibody (4G10, UBI, Lake Placid) and developed by enhanced chemiluminescence (Amersham International, UK) using standard protocols. Arrows indicate position of the EGF-receptor and actin.

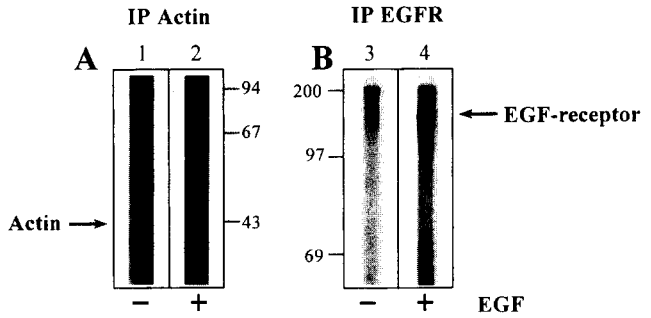

Fig. 4. In vivo actin phosphorylation upon EGF stimulation HER14 cells were starved O/N in DMEM-7.5% FCS and labelled for four hours with [^{32}P]orthophosphate as described in section 2. Both actin and the EGF-receptor were immunoprecipitated from the cells after 10 min of stimulation with 50 ng/ml EGF and the proteins were separated by 10% SDS-PAGE. Arrows indicate phosphorylated actin (A) and EGF-receptor (B).

phosphorylated, the excised band of phosphorylated actin was subjected to phospho-amino acid analysis. Fig. 5A shows a phospho-serine spot of hydrolysed actin while Fig. 5B shows a schematic representation of the position of the three phospho-amino acids. In vivo actin is phosphorylated on serine and not on tyrosine residues upon EGF stimulation, confirming that the phosphorylation of actin is not caused by the EGF-receptor, but by a serine/threonine kinase that becomes activated after EGF stimulation.

4. Discussion

One of the results of stimulation of the EGF-receptor is phosphorylation of various cytoskeletal components. Actin, being a cytoskeletal protein and directly bound to the EGF-receptor is a good candidate for EGF directed phosphorylation. Our results, however, demonstrate that in vivo actin becomes phosphorylated on serine and not on tyrosine residues upon EGF stimulation. In addition we found that membrane bound actin in vitro serves as substrate for a serine/threonine kinase stimulated after EGF addition. The identity of this serine/threonine kinase is not yet known.

The actin found to be phosphorylated is only a very small percentage of total cell actin, and as demonstrated in the in vitro studies is present in the membrane preparations. This suggests that the phosphorylated actin is part of the membrane skeleton and may be present in membrane ruffles. Membrane ruffles appear after stimulation of cells by growth factors such as EGF [22,23]. These ruffles are thought to be a focus of signal transduction events, in that active receptors and tyrosine phosphorylated proteins are highly localised in the membrane ruffles. Various actin binding proteins like, ezrin, spectrin and calpactin II are also recruited to the ruffles and become phosphorylated on both serine, threonine and tyrosine residues upon EGF treatment of cells [6,24]. Thus, another possible function of actin phosphorylation could be that actin phosphorylation is used to create a specific binding site for other proteins.

As previously described, evidence has been obtained that the phosphorylation of actin facilitates actin polymerisation [15]. As such it seems attractive to suggest that the EGF-induced phosphorylation of actin is involved in the EGF-induced actin

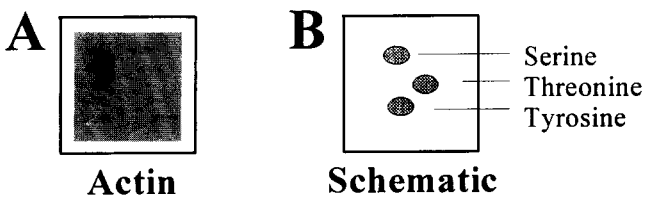

Fig. 5. Phospho-amino acid analysis (PAA) of in vivo phosphorylated actin. Actin phosphorylated in vivo was excised from the gel, TCA precipitated and hydrolysed with HCl as described in section 2. Phosphorylated amino acids were separated in two dimensions on thin layer chromatography plates, and their position compared to migration of cold phospho-amino acid standards. (A) autoradiograph of actin phospho-amino acids after EGF stimulation. (B) schematic representation of the migration pattern of cold phospho-amino acid standards.

polymerisation [8]. Of interest in this respect is the fact that the EGF-induced actin polymerisation is restricted to the cortical cell area [9], and here we have demonstrated that actin phosphorylation is due to a serine kinase present in the membrane fraction (Fig. 1). Thus the EGF-induced actin phosphorylation probably occurs at the same cell location as the actin polymerisation, suggesting that these two processes may be linked to each other. There are several serine/threonine kinases that are activated upon EGF that bind to the membrane such as Raf [25], or casein kinase I [26], thus any of these could be a candidate for delivering the kinase activity that phosphorylates actin. Currently work is underway, using specific inhibitors and immunocomplex kinase assays to determine if any of these kinases can phosphorylate actin in vitro.

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