Epidermal growth factor induces rapid reorganization of the actin microfilament system in human A431 cells

PHILIP J. RIJKEN[1,*], WILLEM J. HAGE[2], PAUL M. P. VAN BERGEN EN HENEGOUWEN[1], ARIE J. VERKLEIJ[1] and JOHANNES BOONSTRA[1]

[1]*Department of Molecular Cell Biology, University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands*
[2]*Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands*

* Author for correspondence

Summary

Double immunofluorescence microscopy reveals that epidermal growth factor (EGF) treatment of A431 cells results in more apparent co-localization of EGF receptor (EGFR) and actin filaments, as compared to control cells. This indicates that EGF induces actin polymerization as well as additional association of the EGFR with similar sites on the membrane-skeleton. We show that immunoprecipitation of the cytoskeleton-linked EGFR after fragmentation of the cytoskeleton results in specific co-precipitation of F-actin and a limited set of other unidentified proteins. Interestingly, EGF treatment of intact cells results in increased immunoprecipitation of cytoskeleton-associated EGFR as well as of F-actin, while actin does not co-precipitate with the non-ionic detergent-solubilized EGFR. These results demonstrate that the cytoskeleton-linked EGFR is associated with the actin microfilament system. EGF induces additional formation of protein complexes, containing the EGFR and F-actin and a limited set of other unidentified proteins. The increased co-precipitation of F-actin is most likely related to EGF-induced actin polymerization, which is specifically associated with the apical cortical microfilament system, as demonstrated by confocal laser scanning microscopy and a phallicidin-binding assay.

Key words: actin, epidermal growth factor, polymerization, confocal laser scanning microscopy.

Introduction

Epidermal growth factor (EGF) is recognized as an important determinant in the regulation of cellular proliferation, both *in vivo* and *in vitro*. EGF exerts its effects in the target cells by binding to the EGF receptor (EGFR), a 170×10^3 M_r transmembrane protein with an extracellular EGF-binding domain and an intracellular protein tyrosine kinase domain. The EGFR-kinase is activated upon EGF binding and is responsible for several early responses, including protein phosphorylations, an increase in phosphatidylinositol turnover, elevation of intracellular free calcium concentration and induction of early gene expression. Activation of the EGFR-kinase ultimately leads to stimulation of DNA synthesis and cell division in most cells (Carpenter and Cohen, 1979; Carpenter and Cohen, 1990; Schlessinger *et al.* 1983).

The presence of at least two sub-classes of EGF binding sites in EGF-responsive cells has been indicated by different experimental approaches (Berkers *et al.* 1991; Boonstra *et al.* 1985; Defize *et al.* 1989; King and Cuatrecasas, 1982). In A431 cells, EGF binding studies performed under equilibrium conditions and analyzed according to the method of Scatchard indicate the presence of two classes of EGFR, the high- and low-affinity EGFRs (Defize *et al.* 1989; King and Cuatrecasas, 1982; Van Bergen en Henegouwen *et al.* 1989). The primary structures of both receptor sub-classes are probably identical because transfection of cells exhibiting no endogenous EGFR with an expression vector containing a single EGFR gene results in expression of both high- and low-affinity receptors (Livneh *et al.* 1986; Prywes *et al.* 1986). Recently, however, evidence has been provided that indicates a functional difference between these EGFR subclasses. The mAb 2E9 was used to block EGF binding to the low-affinity EGFR, while leaving binding to the high-affinity receptors unimpaired (Defize *et al.* 1989). Using these properties it was demonstrated that activation of the EGFR-mediated signal transduction cascade by EGF, occurs through the HA-EGFR population. Other studies have confirmed these findings (Bellot *et al.* 1990).

It is therefore of particular interest that the HA-EGFR is associated with the non-ionic detergent-insoluble cytoskeleton, as demonstrated using various electron-microscopic methods in combination with Scatchard analysis (Roy *et al.* 1989; Van Bergen en Henegouwen *et al.* 1989; Wiegant *et al.* 1986). The cytoskeleton-associated receptors have been shown to retain a functional ligand binding domain and EGF-induced kinase activity (Landreth *et al.* 1985; Roy *et al.* 1989; Van Bergen en Henegouwen *et al.* 1989), both of which are essential for EGF-induced signal transduction (Moolenaar *et al.* 1988). It is therefore tempting to suggest that the cytoskeleton has an important function in signal transduction. Several lines of evidence support this suggestion. First, EGFR-kinase activation leads to phosphorylation of a number of

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cytoskeleton-associated proteins including calpactin, spectrin, ezrin, fodrin and microtubule-associated protein-2 (Akiyama *et al.* 1986; Bretscher, 1989; Fava and Cohen, 1984). Second, in A431 cells several components involved in EGF-induced signal transduction such as phospholipase C (PLC), phosphatidylinositol-kinase, phosphatidylinositol-4-phosphate kinase and diacylglycerol-kinase are associated with the cytoskeleton (Payrastre *et al.* 1991). In addition, protein kinase C as well as diacylglycerol appear to interact with the cytoskeleton (Burn *et al.* 1985; Ito *et al.* 1989). Third, other growth factor receptors are associated with the cytoskeleton. These include the receptors for nerve growth factor and platelet-derived growth factor, illustrating the fact that the EGFR is not unique in this respect (Vale and Shooter, 1983; Zippel *et al.* 1989). Finally, a number of proteins involved in EGF-induced signal transduction have been demonstrated to contain conserved domains thought to play a role in actin binding. Phospholipase Cγ, vinculin, calpactin and spectrin contain such domains (Drubin *et al.* 1990; Tellam *et al.* 1989). These domains have also been identified in non-receptor tyrosine kinases as c-*src* and c-*abl*, both involved in control of cell growth and differentiation (Drubin *et al.* 1990; Hirai and Varmus, 1990; Moran *et al.* 1990). In summary, numerous observations indicate that the cytoskeleton is somehow involved in signal transduction. In this respect it is of interest that EGF has been demonstrated to influence specifically actin organization in A431 cells (Schlessinger and Geiger, 1981) and that EGF induces a substantial increase in EGFR association with the cytoskeleton, as revealed by Scatchard analysis (Van Bergen en Henegouwen *et al.* 1989). In addition, from studies using electron-microscopic methods, it has been suggested that the EGFR is associated with the actin microfilament system (Wiegant *et al.* 1986).

In this paper we have studied in detail the effects of EGF on the organization of the actin-containing microfilament system in human A431 cells. In addition, the cytoskeletal component involved in EGFR interaction with the cytoskeleton has been identified and the effect of EGF treatment on this interaction has been investigated. We demonstrate, using immunofluorescence light microscopy, that EGF causes a significant change in actin microfilament organization, at both 37 °C and at 4 °C. Importantly, the apparent colocalization of EGFR and actin filaments, as observed in control cells, is enhanced at both temperatures following EGF treatment, especially in areas of cell to cell contact and in plasma membrane extrusions. These observations suggest that there is EGF-induced actin filament formation as well as increased association of the EGFR with similar sites on the membrane skeleton. Immunoprecipitation of the cytoskeleton-associated EGFR results in co-precipitation of filamentous actin (F-actin) and some other unidentified proteins, but not of keratin or tubulin, suggesting that the EGFR is associated with actin-containing filaments. Specificity of actin co-precipitation with the cytoskeleton-associated EGFR is indicated by the observation that actin does not co-precipitate with the non-ionic detergent-solubilized receptor. Treatment of intact A431 cells with EGF results in increased immunoprecipitation of the EGFR from the cytoskeletal fraction, but also in increased co-precipitation of F-actin and some of the other proteins. This suggests that EGF induces additional EGFR association with actin-containing filaments as well as the formation of protein complexes containing F-actin, the EGFR and a limited set of other proteins. The increased co-precipitation of F-actin in EGF-treated cells is most likely the result of EGF-induced actin polymerization that is associated specifically with the cortical actin microfilament system, as observed by confocal laser scanning microscopy and a phallicidin-binding assay. This process coincides with the disappearance of stress fibre actin and suggests that there is filamentous actin disassembly elsewhere in the cell.

Materials and methods

Materials

EGF was obtained from Collaborative Research (Waltham, MA, USA). The mAb 2E9 and the polyclonal Ab 281–7 were raised, isolated and characterized as described in detail elsewhere (Defize *et al.* 1989). The mAb 528 (mAb AB-1) was obtained from Oncogene Science Inc. (Manhasset, NY, USA). The actin mAb designated N.350 was from Amersham International (Houten, The Netherlands). Polyclonal anti-cytokeratin-18 was kindly provided by Professor Dr F. Ramaekers (University of Maastricht, The Netherlands). Partially purified actin (approx. 90 % pure), FITC-labelled phallicidin, nitro blue tetrazolium (NBT), 5-bromo-4-chloro-3 indolyl phosphate (BCIP), Triton X-100 and phenylmethylsulfonyl fluoride (PMSF) were from Sigma Chemical Co. (St Louis, MO, USA). Gelatin was from Merck (Darmstadt, FRG). Paraformaldehyde was obtained from BDH Chemicals (Poole, UK). The goat anti-mouse–TRSC (Texas Red) conjugate (IgG +IgM (H+L)), goat anti-mouse–biotin (GAM–biotin) and goat anti-rabbit–biotin (GAR–biotin) were from Jackson Immunoresearch Laboratories Inc. (Westgrove, PA, USA). For immunoprecipitation studies mAb 528 was coupled to Sepharose CL-4B from Pharmacia LKB Biotechnology (Uppsala, Sweden). FCS was from Integro (Zaandam, The Netherlands) and DME was from Gibco Europe B.V. (Paisley, Scotland). The streptavidin–alkaline phosphatase conjugate (SAPC) was from BRL Life Technologies Inc. (Gaithersburg, MD, USA).

Cell culture

A431 human epidermoid carcinoma cells (passage 10–40) were grown in DME supplemented with 7.5 % FCS in a 7 % CO_2 humidified atmosphere. Cells were allowed to spread and adhere to the substratum for at least 48 h and were cultured until small groups of cells had formed (8–24 cells per colony) and no more then 50 %–80 % of the substratum was covered with cells. For all experiments cell culture material from Costar (Cambridge, MA, USA), or glass coverslips were used.

Double immunofluorescence microscopy

A431 cells were cultured on glass coverslips as described under cell culture (above). Cells were treated with DME–Hepes with EGF (80 ng ml^{-1}) or buffer alone for 6 min at 37 °C, or for 2.5 h at 4 °C, quickly washed in PBS containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$, and fixed for 30 min in 3.5 % formaldehyde in PBS. Next, cells were washed in PBS–glycine (PBS supplemented with 50 mM glycine) and PBS–gelatin (PBG: PBS supplemented with 0.1 % gelatin) five times, 2 min each, and labelled with phallicidin–FITC (2 μg ml^{-1}) in PBG and/or 2E9 (33 μg ml^{-1}) in PBG for 1 h at room temperature. 2E9 has been demonstrated to recognize specifically the EGFR and binds to this receptor approximately in a 1:1 molar ratio (Defize *et al.* 1989). Following first antibody (2E9) or phallicidin–FITC incubation, cells were washed with PBG and incubated with secondary goat anti-mouse–TRSC conjugate in PBG for 1 h at room temperature and subsequently intensively washed with PBG. Labelled cells were embedded in Mowiol and photographed using Kodak Tri-X-Pan film on a Wild-Leitz epi-illuminance microscope with 63× oil objective lens.

Immunoprecipitation of the EGFR

A431 cells were treated with binding buffer (DME–Hepes containing 0.1 % BSA) with or without EGF (80 ng ml^{-1}) for 2.5 h at 4 °C. Cells were washed once with binding buffer and extracted for 10 min with non-ionic detergent, using extraction buffer

(10 mM Hepes, 1 mM PMSF, 1 mM $MgCl_2$) containing 0.5 % Triton X-100. Supernatant containing solubilized EGFR was collected and the remaining cytoskeletons were gently washed twice with extraction buffer (omitting the Triton X-100). Cytoskeletons were scraped and homogenized (25 times in a Potter homogenizer) and centrifuged once for 2.5 min at 500 *g* to remove nuclei. The eluate containing the homogenized cytoskeleton was collected (referred to as cytoskeleton). Immunoprecipitation was performed on 150 μg of total protein from extract and cytoskeleton in identical volumes (1.5 ml), using a molar excess of anti-EGFR mAb 528 (1.5 μg mAb 528). To reduce aspecific binding, samples were cleared with Sepharose CL-4B for 30 min prior to the immunoprecipitation. For optimal recovery preadsorbed mAb 528-coupled Sepharose CL-4B was added to extract and cytoskeleton fractions, respectively. Immunoprecipitates were washed extensively with extraction buffer (8 times in 1.5 ml). After each wash Protein A–Sepharose was spun down by centrifugation for 1 min at 500 *g* and immunoprecipitates were finally dissolved in 30 μl sample buffer. Samples were boiled for ~10 min at 95°C and proteins were separated by SDS–PAGE on 10 % gels. Subsequently, gels were silver stained or transferred to nitrocellulose membranes. Proteins were identified by incubation of blots with the anti-actin mAb N.350, polyclonal anti-keratin 18, or the anti-EGFR polyclonal rabbit antibody 2–81–7. Blots were incubated with GAM–biotin or GAR–biotin, respectively, SAPC and a phosphatase–substrate mixture of NBT and BCIP.

Phallicidin–FITC binding assay

Cells were treated with EGF (80 ng ml^{-1}) in buffer (DME-supplemented with 10 mM Hepes) or with buffer alone and were fixed in 3.5 % formaldehyde in PBS for 30 min, washed in PBS–glycine and PBG five times, 2 min each, and labelled for 1 h with phallicidin–FITC at room temperature. After several washes in PBG and PBS–glycine, respectively, cells were dissolved in 1.5 M NaOH for 4 h at a temperature of 45°C. This solution was buffered by addition of PBS and set at pH 8.0±0.1. Solutions of EGF-treated and control cells were analyzed for relative fluorescence emission (RFE) in a 2 ml capacity, 10 mm pathlength cuvette at an optimal excitation wavelength of 495 nm and an optimal emission wavelength of 520 nm, with a bandwidth of 5 nm on an SLM Aminco SPF 500 spectrofluorometer. Data were corrected for background emission (<0.5 % of total signal), originating from sources other than phallicidin–FITC, using unlabelled control samples. Next, solutions were analyzed for absorption at 260 nm on a Beckman spectrophotometer (model 24) to correct for differences in cell number. Relative F-actin content (RFC) is the ratio of RFE from EGF-stimulated samples to RFE of buffer-treated samples, after correction for cell number using the absorption at 260 nm. Subsequently, the RFC is calculated as the ratio of:

$$\frac{\text{RFE of EGF-treated cells/absorption at 260 nm}}{\text{RFE of control cells/absorption at 260 nm}}.$$

Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) was performed on formaldehyde-fixed A431 cells, prepared as described for conventional double immunofluorescence microscopy with a Bio-Rad 500 instrument on a Zeiss Axioplan microscope. This technique allows for optical sectioning (~1 μm section thickness) parallel or perpendicular to the cell substratum. For high-resolution imaging, a 63× oil objective (NA 1.4) was used. Optical sections were taken at intervals of ~1 μm parallel to the cell substratum. For alternative observation of cellular F-actin distribution, optical sections of ~1 μm thickness were taken perpendicular to the cell surface. Sections were produced by line-scanning.

Results

Co-localization of EGF receptors and filamentous actin

Co-localization between the EGFR and actin filaments in A431 cells at the electron-microscopic level (Wiegant *et al.* 1986) suggested that the actin-containing microfilament system may be involved in EGFR interaction with the cytoskeleton. Therefore, we examined the consequence of both drastic EGF-induced actin filament reorganization (Schlessinger and Geiger, 1981) and EGF-induced EGFR association with the cytoskeleton (Van Bergen en Henegouwen *et al.* 1989) on co-localization, using double immunofluorescence microscopy.

A431 cells were fixed using 3.5 % formaldehyde and subsequently labelled with either phallicidin–FITC (Fig. 1A,E) or anti-EGFR mAb 2E9 (Fig. 1B,F). Phallicidin is known for its specific high-affinity binding to F-actin (Wulf *et al.* 1979) and is widely used for the visualization of F-actin in mammalian cells and in addition for quantification of the cellular F-actin content of cells in suspension

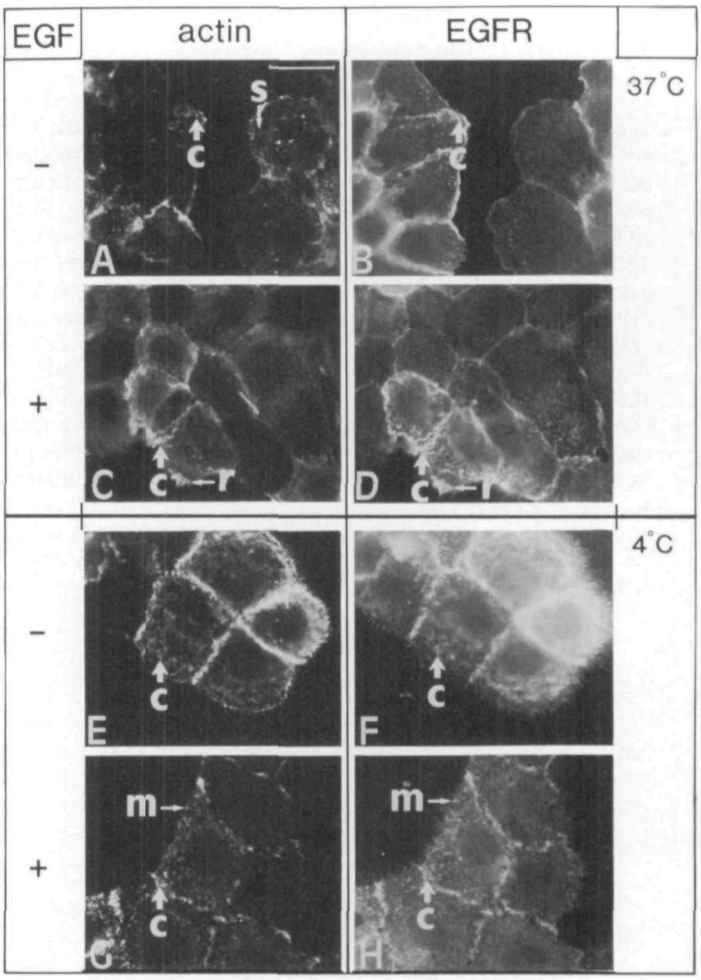

Fig. 1. The EGFR colocalizes with the peripheral cortical actin microfilament system in human A431 carcinoma cells. Cells were incubated with EGF (80 ng ml^{-1}; C,D,G,H) or with buffer alone (A,B,E,F) for 6 min at a temperature of 37°C (A,B,C,D), or for 2.5 h at a temperature of 4°C (E,F,G,H,). Cells were fixed with 3.5 % formaldehyde and labelled with phallicidin–FITC (2 μg ml^{-1}; A,C,E,G) or mAb 2E9 (33 μg ml^{-1}; B,D,F,H,), as described in Materials and methods. Co-localization of actin filaments with the EGFR (indicated by arrows and marked C) is indicated under both temperature conditions. Control experiments include background fluorescence, non-specific secondary antibody binding and cross-reactivity and were negative (not shown). Bar in A, 25 μm. Results shown are representative of three independent experiments. r, ruffling; m, microvilli; s, stress fibre.

(Cassimeris *et al.* 1990; Hall *et al.* 1988; Rao *et al.* 1990). At 37°C, F-actin is primarily associated with stress fibres (marked by s) and also with areas of cell-to-cell contact in control cells (Fig. 1A). To allow comparison with other data described in this paper, cells were transferred to 4°C. Incubation of cells at 4°C for 2.5 h prior to fixation resulted in the appearance of shorter F-actin bundles and small regions of relatively high F-actin staining (Fig. 1E). The EGFR, labelled with the mAb 2E9 and goat anti-mouse TRSC conjugate, appears randomly distributed over the plasma membrane (Fig. 1B). Incubation of cells at 4°C for 2.5 h resulted in the appearance of regions containing relatively high amounts of EGFR (Fig. 1F). Comparison of Fig. 1A with B and of Fig. 1E with F reveals that some of the areas displaying relatively high F-actin staining also contain higher amounts of EGFR (indicated by arrows and marked with C). This is especially clear in cells incubated at 4°C for 2.5 h. Stress fibres, however, did not exhibit increased EGFR labeling.

To determine the effect of EGF treatment of A431 cells on EGFR and F-actin distribution, cells were incubated with 80 ng ml^{-1} EGF for 6 min at 37°C (Fig. 1C,D), or for 2.5 h at 4°C (Fig. 1G,H). Such treatment causes extensive ruffling at 37°C and formation of microvilli at 4°C (Fig. 1C,D and G,H, marked r and m). Remarkably, compared to control cells, F-actin staining is now associated primarily with these plasma membrane extrusions and areas of cell-to-cell contact (Fig. 1C,G). The EGFR (Fig. 1D,H) also becomes non-randomly distributed in EGF-treated cells at both temperatures, with relatively high receptor numbers also in association with plasma membrane extrusions (ruffles, microvilli). EGF treatment of A431 cells results in clearly more pronounced co-localization of EGFR and F-actin, especially in membrane ruffles, microvilli and areas of cell-to-cell contact. Importantly, no co-localization was observed between EGFR and microtubules or keratin filaments (not shown). Control experiments included background fluorescence, non-specific secondary antibody binding and cross-reactivity. These observations clearly demonstrate that co-localization of the plasma membrane-localized EGFR with actin filaments is enhanced during EGF treatment, both at 37°C and at 4°C.

Co-precipitation of actin with the cytoskeleton-associated EGFR

We examined the consequence of EGF treatment of intact cells on EGFR interaction with the cytoskeleton by immunoprecipitation of the cytoskeleton-associated EGFR. After extraction of A431 cells with 0.5% Triton X-100, the cytoskeleton was fragmented and homogenized to preserve existing molecular interactions between receptor and cytoskeleton, but to allow immunoprecipitation of the EGFR. The anti-EGFR mAb 528 was used to immunoprecipitate the EGFR from the fragmented cytoskeleton and supernatant, respectively. Importantly, each immunoprecipitation was performed using 150 μg of total protein and excess mAb 528. Immunoprecipitated proteins were separated by SDS–PAGE and subsequently immunoblotted or silver-stained.

The EGFR is identified by immunodetection with the anti-EGFR polyclonal antibody 2–81–7 (Fig. 2A) and is

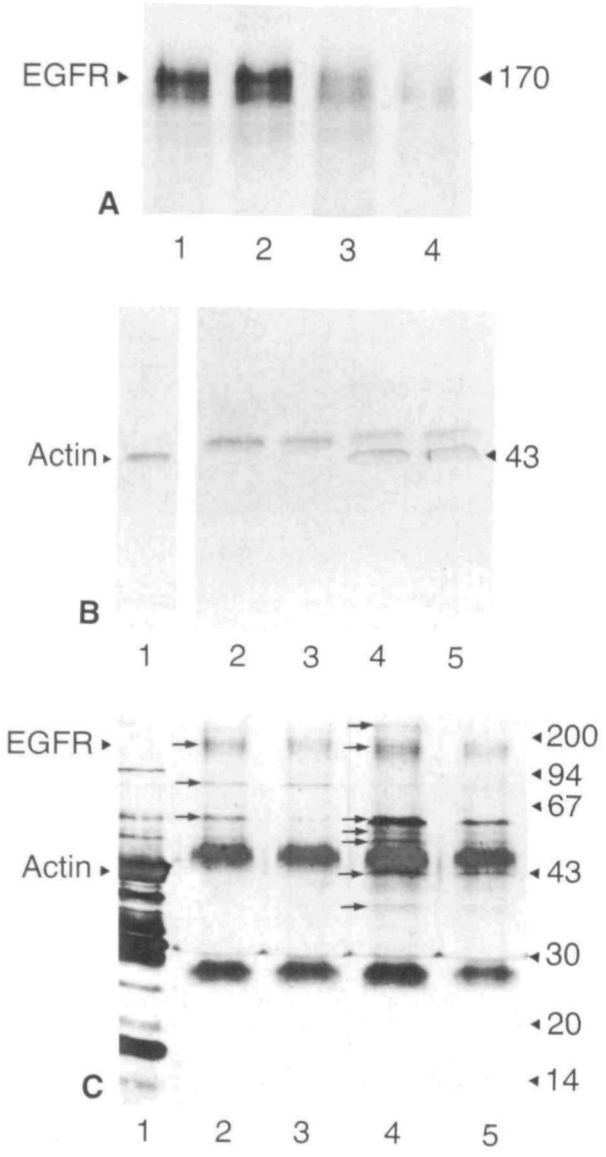

Fig. 2. Actin specifically co-precipitates with the cytoskeleton-linked EGFR, not with the Triton X-100-solubilized EGFR. A431 cells were treated with EGF (80 ng ml^{-1}) for 2.5 h. Cells were extracted for 10 min with Triton X-100, and cytoskeleton and supernatant were collected as described in Materials and methods. From each fraction the EGFR was immunoprecipitated using excess mAb 528. Proteins were separated by SDS–PAGE and transferred to nitrocellulose paper. Results are representative of four independent experiments. M_r values on the right are $\times 10^{-3}$. (A) Lanes 1 and 2 represent immunoblots of immunoprecipitated EGFR from the supernatant of EGF-treated (80 ng ml^{-1}) and control cells, respectively; lanes 3 and 4 represent immunoblots of immunoprecipitated EGFR of the cytoskeletal fraction of EGF-treated and control cells, respectively. Immunoblotting was performed using the anti-EGFR polyclonal antibody designated 2–81–7. (B) Lane 1 represents immunoblot of partially purified actin; lanes 2 and 3 represent immunoblots of immunoprecipitated EGFR of the supernatant of EGF-treated and control cells, respectively; lanes 4 and 5 represent immunoblots of immunoprecipitated EGFR of the cytoskeletal fraction of EGF-treated and control cells, respectively. Immunoblotting was performed with the anti-actin mAb N.350. (C) Silver-stained gel of immunoprecipitated EGFR and associated proteins, present in the supernatant (lanes 2 and 3) or in the cytoskeletal fraction (lanes 4 and 5) of A431 cells. Cells were treated with buffer alone (lanes 3 and 5) or with EGF for 2.5 h (80 ng ml^{-1}; lanes 2 and 4). Similar gels were used for protein transfer to nitrocellulose paper.

present as approximately $170\times10^3 M_r$ and $150\times10^3 M_r$ proteins in all fractions. The appearance of a double band on the immunoblot is probably due to limited protein degradation (Cohen *et al.* 1982). From the supernatant substantially more EGFR was immunoprecipitated (Fig. 2A, lanes 1,2), as compared to the fragmented cytoskeleton (Fig. 2A, lanes 3,4). This is due to the fact that ~90–95% of the total EGFR resides in the supernatant after Triton X-100 extraction (Van Bergen en Henegouwen *et al.* 1989). A similar experiment was performed using cells preincubated with 80 ng ml^{-1} EGF at 4°C for 2.5 h. This resulted in increased EGFR immunoprecipitation from the cytoskeletal fraction (Fig. 2A, lane 3 *versus* lane 4). This is in agreement with data obtained by Scatchard analysis under similar experimental conditions, demonstrating that EGF increases the amount of cytoskeleton-associated EGFR (Van Bergen en Henegouwen *et al.* 1989).

Interestingly, a protein with an apparent molecular weight of 43×10^3 co-precipitates with the EGFR in the cytoskeletal fraction (Fig. 2C, lanes 4 and 5). This protein co-migrates with partially purified actin (Fig. 2B lane 1). By immunoblotting using anti-actin mAb N.350 (Fig. 2B, lanes 4 and 5), a strong signal was obtained, indicating that this protein is actin. The $50\times10^3 M_r$ bands in Fig. 2B (lanes 2,3,4,5) are due to immunoreactivity of the heavy chain of the mAb 528 towards the polyclonal goat anti-mouse–biotin used for immunodetection of the mouse anti-actin mAb. Actin co-precipitates only with the cytoskeleton-associated EGFR, indicating that this receptor is associated with the F-actin-containing cytoskeleton. EGF treatment of intact cells results in increased co-precipitation of actin (Fig. 2C, lane 5 *versus* lane 4). Actin does not co-precipitate with the detergent-solubilized EGFR (Fig. 2C, lanes 2 and 3), indicating that this receptor population is not associated with actin.

To investigate whether other proteins co-precipitate with the EGFR from both fractions, immunoprecipitated proteins were separated by SDS–PAGE and gels were silver stained (Fig. 2C). Several proteins with approximate relative molecular masses of 36, 43, 53, 57, 61×10^3 and a high M_r protein ($>200\times10^3$) co-precipitate with the cytoskeleton-linked EGFR (Fig. 2C, lanes 4 and 5, indicated by arrows). In contrast, the detergent-solubilized EGFR most noticeably co-precipitates with three proteins with approximate M_r of 61×10^3 and 85×10^3 (Fig. 2C, lanes 2 and 3, indicated by arrows). This demonstrates that the cytoskeleton-associated and the non-ionic detergent-solubilized EGFR populations are associated with distinct sets of proteins. EGF treatment of A431 cells appears not to lead to association of additional proteins with the complex, nor does it dissociate specific proteins from the complex (Fig. 2C, lanes 2 and 4 *versus* lane 3 and 5). EGF treatment increases the amount of protein co-precipitating with the EGFR, as compared to control cells, most evidently in the cytoskeletal fraction, as already indicated for actin. Semiquantitative analysis of the gels confirmed this, even after correction for the differences in mAb 528 light chain staining in the gels (excess mAb 528 was used). Most other proteins in the immunoprecipitates, but not the $85\times10^3 M_r$ protein, are more abundantly present in the immunoprecipitates of EGF-treated cells.

Under the conditions used, most tubulin is removed from the cytoskeletal fraction after Triton X-100 extraction (not shown). Thus, tubulin does not appear obligatory for EGFR interaction with the cytoskeleton. Of the different cytokeratins expressed in A431 cells, keratin 18 is one of the most abundant (Moll *et al.* 1982). Keratin 18 could not be detected in the immunoprecipitates of control or EGF-treated cells by using an anti-keratin 18 polyclonal antiserum (not shown). This indicates that keratin 18 is not involved in EGFR association with the cytoskeleton. These results suggest that EGF increases EGFR association specifically with actin-containing filaments, but also leads to increased formation of protein complexes, containing F-actin, the EGFR and a limited set of other proteins.

EGF induces actin polymerization

EGF treatment of intact A431 cells leads to an increase in EGFR association with the actin-containing microfilament system. However, it also increases co-precipitation of F-actin and other unidentified proteins. It can be speculated that these processes are related. For this reason we characterized in detail EGF-induced changes in organization of the actin microfilament system, using an adapted phallicidin–FITC binding assay as described by Howard and Oresajo (1985). This assay determines the relative F-actin content (RFC) as described briefly below.

A431 cells were fixed and labelled with phallicidin–FITC (2 μg ml^{-1}), washed extensively and lysed in 1.5 M NaOH for 4 h at 45°C. In this way all bound phallicidin–FITC is collected along with all solubilized cells. However, the pH of the phallicidin–FITC-containing lysate is 13.9. At this pH, a marked decrease in the relative fluorescence emission (RFE) is measured. The RFE returns to control levels if the pH of the lysate is set with HCl at 8.0±0.1. Thus, the decrease in RFE is due to pH dependency of fluorescence emission, not to damage to the fluorescent group. The relationship between the F-actin content and the RFE was investigated. Increasing F-actin content of the samples was obtained by lysis of increasing numbers of labelled cells with 1.5 M NaOH. After correction of all data for background fluorescence, as determined using unlabelled control cells, it was found that the RFE is directly proportional to the F-actin content. However, the minimum cell number for accurate measurements appears to be $\sim0.5\times10^5$. By using significantly lower cell numbers, background fluorescence is approached. For phallicidin binding assays 2×10^5 to 3×10^5 cells were used and all data were corrected for background fluorescence, using unlabelled samples. Differences in cell number per sample as well as loss of cells during execution of the experiment, may give rise to significant errors in determination of the RFC. To correct for such differences, the absorption of the lysates at 260 nm was determined. It was found that this parameter is, within a defined range, directly proportional to the cell number. Finally, it was determined that with increasing phallicidin–FITC concentrations, fluorescence emission increases linearly. These results demonstrate that this phallicidin–FITC binding assay is suitable for quantification of the RFC in adherent cells.

To detect EGF-induced changes in F-actin formation in A431 cells, the RFC was monitored. As shown in Fig. 3, addition of EGF to A431 cells at 37°C leads to a rapid increase in RFC. Within 3 min, the cellular F-actin content increases by almost 17% and remains constant for 3–5 min. After 15 min the RFC was more than 30% higher, as compared to control cells. RFC values do not rise significantly higher after prolonged incubation with EGF (not shown). Maximal actin polymerization occurs in the first 3 min after EGF addition. However, the time resolution of each experiment was such that some information may be lost after determination of the

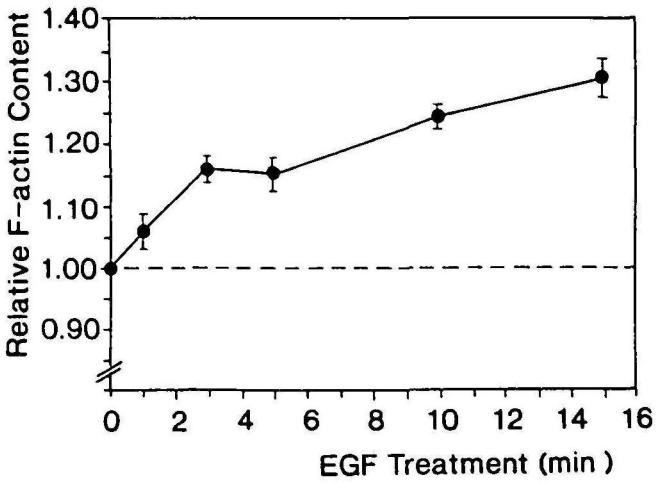

Fig. 3. EGF induces rapid increase in the relative F-actin content. The relative F-actin content (RFC) was determined as defined in Materials and methods. A431 cells were treated with EGF (80 ng ml^{-1}) or buffer alone, at a temperature of 37 °C for the times indicated. Cells were fixed with formaldehyde (3.5 %) and incubated with phallicidin–FITC as described in Materials and methods. The graph represents average data from five independent experiments. (---) The RFC of control cells. For each assay the RFC was determined on ~2.5×10^5 to ~3×10^5 cells per experiment (n=5; bars represent S.E.M.).

average RFC of several experiments. Often, a transient increase in RFC in the first 5 min of EGF treatment was observed in individual experiments. Alternative processes that lead to actin depolymerization may explain this. At 4 °C, similar results were obtained, although the RFC increased with ~20 % after 2.5 h of EGF treatment (not shown). These results demonstrate that EGF causes a rapid increase in F-actin content of A431 cells. These data, combined with the results from figures 1 and 2, suggest that EGF-induced actin polymerization and EGFR association with the cytoskeleton occur at similar locations close to the plasma membrane. Therefore, the intracellular sites of EGF-induced actin polymerization were determined in detail by confocal laser scanning microscopy.

EGF-induced actin polymerization occurs specifically at the apical membrane cytoskeleton

In A431 cells actin-containing microfilament bundles are observed by conventional immunofluorescence microscopy at 37 °C (Fig. 1). Optical sectioning by confocal laser scanning microscopy (CLSM) parallel to the cell substratum reveals that these bundles are located predominantly in the basal region of the cell, as well as in a belt at the cell periphery (Fig. 4A1 and A2; stress fibre, S). In contrast, the apical region of adherent A431 cells is usually devoid of stress fibre actin (Fig. 4A4). Here, a faint peripheral F-actin staining, often associated with microvilli, can be observed (Fig. 4A4, M). Remarkably, EGF treatment results in a gradual increase in F-actin formation at the apical cell surface (Fig. 4B4), as well as in gradual loss of most stress fibers at the cell basis (Fig. 4B1). After two minutes of EGF treatment, plasma membrane ruffles appear (marked with R) with relatively high F-actin content. This process is accompanied by a gradual rounding of the cell, characterized in detail elsewhere (Chinkers *et al.* 1979; Rijken *et al.* 1991). After 10 min, intense F-actin staining is found at the apical cell surface in most cells (Fig. 4C4), while most stress fibres have disappeared (Fig. 4C1). Areas of cell-to-cell contact remain intensively labelled under all conditions.

To determine F-actin distribution in the cell in detail, optical sections were taken perpendicular to the cell substratum (Fig. 5A–5D). In control cells most F-actin is localized at the basal plasma membrane (indicated by V) and in areas of cell-to-cell contact (Fig. 5A). Two minutes

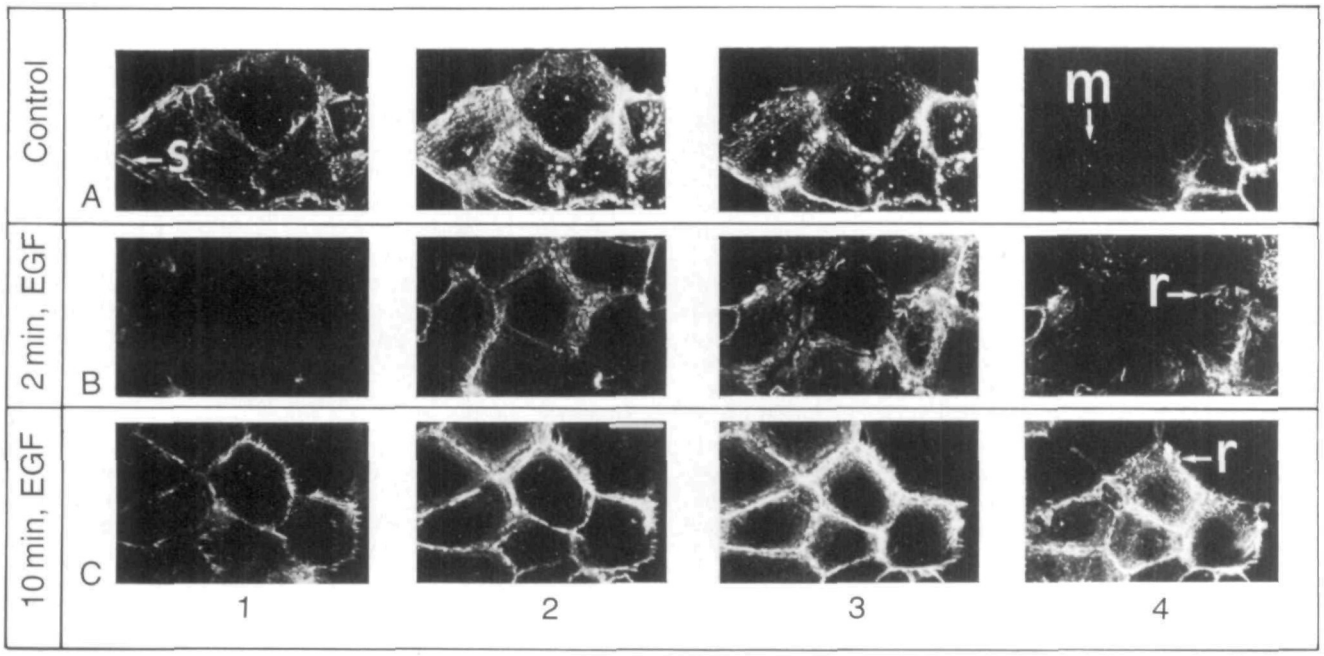

Fig. 4. EGF induces rapid actin filament assembly in the cortical actin microfilament system and disappearance of actin stress fibres. CSLM images represent parallel optical sections of ~1.0 μm at distances of ~0, 1, 2 and 3 μm from the cell substratum (1, 2, 3 and 4, respectively). Cells were treated with 80 ng ml^{-1} EGF for 2 min (B) and 10 min (C), or with buffer alone (A) at 37 °C. Bar in C2, 25 μm. Results are representative of three independent experiments. See Fig. 1 legend for explanation of labels.

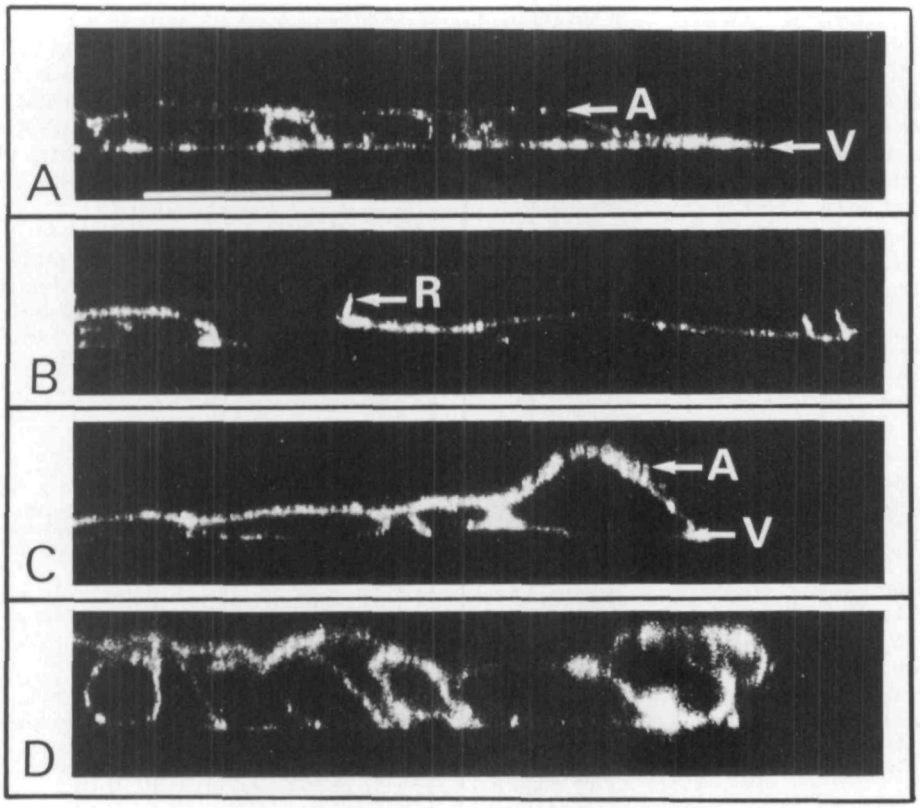

Fig. 5. EGF induces actin polymerization in the apical membrane skeleton, while F-actin disappears from ventral regions of the cell. Optical sections of ~1 μm were taken perpendicular to the cell substratum. Cells were treated with buffer alone (A) or with EGF (80 ng ml^{-1}) for 2 min (B), 5 min (C) and 10 min (D), respectively, under identical conditions to those in Fig. 4. Results are representative of three independent experiments. Bar in A, 25 μm. Results are representative of three independent experiments. V, basal region; A, apical region; R, ruffle.

of EGF treatment results in the disappearance of basal F-actin staining (Fig. 5B) and rapid actin filament formation in the apical region (indicated by A). Especially from these latter data, it is evident that EGF-induced actin polymerization is restricted to specific sites at the apical membrane-skeleton, not at random locations throughout the cell as can be observed in cells treated with EGF for 5 to 10 min (Fig. 5C and D).

Discussion

In this study we demonstrate a co-localization between the EGF receptor (EGFR) and actin microfilaments in human A431 cells, using double immunofluorescence microscopy. This co-localization is even more prominent in cells pretreated with EGF and was found to occur at 37 °C and at 4 °C. An attractive hypothesis to explain the more pronounced co-localization of EGFR and F-actin in EGF-treated cells is that it results from increased F-actin formation and additional association of the EGFR with similar sites on the cytoskeleton. The observations that immunoprecipitation of the EGFR is increased in EGF-treated cells, while EGF treatment also leads to co-precipitation of increased amounts of actin and several other unidentified proteins, support this hypothesis. The specificity of actin co-precipitation with the EGFR is indicated by the fact that neither tubulin nor keratin 18 were detected in immunoprecipitates from either fraction, while actin only co-precipitated with the cytoskeleton-associated EGFR, not with the detergent-solubilized EGFR. These results indicate that the EGFR is associated specifically with the actin microfilament system and that EGF induces additional formation of protein complexes containing the EGFR, F-actin and other unidentified proteins. Furthermore, EGF not only causes changes in the F-actin distribution but also induces a significant increase in actin polymerization. Since stress fibres apparently disappear upon EGF addition, the EGF-induced increase in cellular F-actin content must be due to local actin polymerization. Using CLSM we demonstrate that the EGF-induced actin polymerization is restricted to the apical membrane-skeleton.

An intriguing question concerns the mechanism underlying the observed increase in EGFR association with the actin microfilament system. It has been suggested that clustering of transmembrane proteins leads to an increased affinity of these proteins for cytoskeletal elements (Brandts and Jacobson, 1983). EGF treatment of A431 cells results in clustering (Van Belzen *et al.* 1988) and dimerization (Defize *et al.* 1989) of the EGFR, while clustering of the EGFR results in an increase in the number of cytoskeleton-associated EGFR (Van Belzen *et al.* 1990). Alternatively, EGF-induced receptor clustering and dimerization may lead to direct association between the cytoskeleton-linked EGFR and the plasma membrane-localized EGFR. In this way, the newly associated receptors will not be directly linked to actin filaments. A third possibility is that the EGF-induced increased submembranous actin filament formation is directly related to additional EGFR association with these filaments, not necessarily involving clustering or dimerization. In this respect it is of interest to note that we were able to demonstrate recently a direct interaction between purified EGFR and purified F-actin (unpublished).

As to the underlying molecular mechanism responsible for local actin polymerization in response to EGF treatment in A431 cells, some clues have been obtained by other laboratories. Regulation of actin polymerization is probably mediated by integrated action of phospholipid- and calcium-dependent actin-binding proteins (ABPs), including cofilin, destrin, gelsolin and profilin (Forscher,

1989; Lassing and Lindberg, 1985; Pollard and Cooper, 1986; Yonezawa *et al.* 1990), although direct correlations have sometimes been difficult to establish (Eberle *et al.* 1990; Southwick and Young, 1990). Local actin polymerization occurs as a consequence of release of globular actin (G-actin) from G-actin binding proteins such as profilin, caused by competition of specific phospholipids for the G-actin binding site on profilin. In addition, increase in intracellular calcium concentration may activate calcium-dependent actin filament-severing proteins such as gelsolin. Both increased phosphatidylinositol turnover and increased intracellular free calcium concentration have been demonstrated to occur in A431 cells in response to activation of the EGFR kinase by EGF (Moolenaar *et al.* 1988; Walker and Pike, 1987). These processes may lead to the observed reorganization of the actin microfilament system described in this paper, according to the principles described above. Rapid ligand-induced actin reorganization and/or filamentous actin formation is not restricted to the system investigated in this study. It has been demonstrated in various cell types in response to nerve growth factor, the chemotactic peptide fMet-Leu-Phe and cyclic AMP (Cassimeris *et al.* 1990; Hall *et al.* 1988; Howard and Oresajo, 1985; Paves *et al.* 1990). Also, in unstimulated cells substantial actin polymerization and depolymerization occurs within minutes. In fibroblasts, rapid actin subunit incorporation is restricted to cortical regions of the cell, most noticeably in lamellipodia and microspikes (Okabe and Hirokawa, 1989).

An important issue concerns the biological relevance of the observed effects of EGF. We have demonstrated that EGF treatment of A431 cells leads to increased formation of submembranous F-actin, as well as to increased formation of protein complexes, containing the EGFR, F-actin and other unidentified proteins. It is tempting to speculate that the actin-containing microfilament system plays a regulatory role in EGF-induced signal transduction. This speculation is based upon several observations. First, the organization of the actin microfilament system is clearly linked with changes in phosphatidylinositol turnover. It has been demonstrated *in vitro* that interaction of profilactin complexes with phosphatidylinositol 4,5-biphosphate (PtdIns(4,5)P_2) results in dissociation of actin (Lassing and Lindberg, 1985). Subsequent binding of profilin to PtdIns(4,5)P_2 inhibits its hydrolysis by phospholipase C and therefore causes partial inhibition of PtdIns(4,5)P_2-mediated signal transduction (Goldtschmidt-Clermont *et al.* 1988). A second line of evidence in favour of a regulatory role for the cytoskeleton in signal transduction, is obtained by the observations that a wide variety of cytoskeleton-associated proteins, such as calpactin, spectrin, ezrin and others, are substrates of the EGFR kinase (Akiyama *et al.* 1986; Bretscher, 1989; Fava and Cohen, 1984). Some of these are clearly involved in signal transduction. For example, calpactin has been demonstrated to inhibit phospholipase A2 action in arachidonic acid metabolism (Brugge, 1986). In addition, the lipocortins have been indicated as inhibitors of phospholipase Cγ, thereby down-regulating signal transduction (Machoczeck *et al.* 1989). Compelling evidence in favour of a regulatory role for the cytoskeleton in signal transduction comes from the observation that several key enzymes in EGF-induced signal transduction have been demonstrated to be associated with the cytoskeleton, such as diacylglycerol-kinase, phospholipase C, phosphatidylinositol kinase, phosphatidylinositol 4-phosphate kinase (unpublished). The actin-containing microfilament system may provide a matrix to permit the organization of signalling molecules, their targets and regulators at similar sites in the cell. In this way the cytoskeleton may provide both positive and negative regulatory circuits for signal transduction. Local EGF-induced formation of protein complexes containing the EGFR, F-actin and other proteins fits well within this hypothesis.

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