The EGF Receptor is an Actin-binding Protein

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Abstract. In a number of recent studies it has been shown that in vivo part of the EGF receptor (EGFR) population is associated to the actin filament system. In this paper we demonstrate that the purified EGFR can be cosedimented with purified filamentous actin (F-actin) indicating a direct association between EGFR and actin. A truncated EGFR, previously shown not to be associated to the cytoskeleton, was used as a control and this receptor did not cosediment with actin filaments. Determination of the actin-binding domain of the EGFR was done by measuring competition of either a polyclonal antibody or synthetic peptides on EGFR cosedimentation with F-actin. A synthetic peptide was made homologous to amino acid residues 984–996 (HL-33) of the EGFR which shows high homology with the actin-binding domain of *Acanthamoeba* profilin. A polyclonal antibody raised against HL-33 was found to prevent cosedimentation of EGFR with F-actin. This peptide HL-33 was shown to bind directly to actin in contrast with a synthetic peptide homologous to residues 1001–1013 (HL-34). During cosedimentation, HL-33 competed for actin binding of the EGFR and HL-34 did not, indicating that the EGFR contains one actin-binding site. These results demonstrate that the EGFR is an actin-binding protein which binds to actin via a domain containing amino acids residues 984–996.

THE EGF receptor (EGFR)[1] is a transmembrane glycoprotein of 170 kD with an extracellular EGF binding domain, a single hydrophobic membrane spanning stretch, and an intracellular tyrosine kinase domain (33). Binding of EGF to the receptor leads to dimerization of the receptor and subsequent activation of the tyrosine kinase (30, 31, 42), thereby catalyzing tyrosine phosphorylation of several protein substrates including the receptor itself (16). Activation of the receptor kinase results in a cascade of biochemical and physiological responses, finally leading to stimulation of DNA synthesis in most cells (7). The tyrosine kinase activity was demonstrated to be essential for stimulation of all known early and late responses involved in EGF-induced mitogenic signaling (14, 22, 26).

EGF binding studies revealed the presence of at least two classes of EGFR differing in their affinity of EGF binding, (3, 4, 18), although no evidence is available for heterogeneity of the EGFR primary structure. The low affinity receptors are usually more abundant, but the high affinity class appears to be primarily responsible for induction of the cellular responses to EGF, as deduced from studies in which antibodies were used that specifically inhibited binding of EGF to the low affinity but not to the high affinity receptors (11). Given this major role for the high-affinity class of receptors in EGF-induced signal transduction, it is of particular interest to note that the high affinity receptors are associated to the cytoskeleton (21, 29, 35, 36, 37, 41). It is therefore tempting to suggest that the cytoskeleton has an important function in EGF-induced signal transduction.

This suggestion is supported by the observation that EGFR tyrosine kinase activation leads to phosphorylation of several cytoskeleton-associated proteins, such as the actin-binding proteins annexin I, vinculin, and spectrin (1, 6, 12, 15). Furthermore, several components involved in the EGF-induced signal transduction, among them phospholipase C, phosphatidylinositol (PtdIns) kinase, phosphatidylinositol(4)phosphate (PtdIns(4)P) kinase, and diacylglycerol kinase, have been demonstrated to be associated to the cytoskeleton of A431 cells, in particular to the actin microfilament system (24). Finally, using double-immunofluorescence labeling, it has been demonstrated that the EGFR colocalizes with actin filaments, both in control and EGF-stimulated cells (28, 38). Altogether these observations point to a close interaction between the EGFR and the actin microfilament system.

Support for this interaction was subsequently obtained by selective extraction of cytoskeletons of A431 cells. It was shown that 90% of the cytoskeleton-associated EGFR population was removed from the cytoskeleton together with the actin filamentous system, while during several cycles of poly- and depolymerization of actin isolated from A431 cells, the EGFR sedimented together with polymerized actin (38). Furthermore, it was demonstrated that immunoprecipitation of cytoskeleton-associated EGFR resulted in a specific coprecipitation of actin, while actin did not coprecipitate with nonionic detergent-solubilized EGFR (28). However, so far it is not clear whether the interaction of the EGFR with actin filaments is mediated by other actin-binding proteins,

1. *Abbreviations used in this paper*: EGFR, EGF receptor; PtdIns, phosphatidylinositol; PtdIns(4)P, PtdIns (4) phosphate.

as reported to occur for other cytoskeleton-associated receptors (9, 45), or whether the EGFR binds directly to actin.

We have addressed this question by studying the association of the purified EGFR with purified actin in vitro. We demonstrate that the EGFR specifically binds to polymerized actin with no other proteins involved. EGF receptors contain one actin-binding site which is located between amino acid residues 984–996 of the intracellular domain.

Materials and Methods

Cell Culture

A431 epidermoid carcinoma cells were grown in DME (Gibco, Paisley, Scotland) supplemented with 7.5% FCS (Integro, Zaandam, the Netherlands) in 1,750 cm^2 roller bottles (Falcon, Becton Dickinson Labware, UK) to confluency.

EGF Receptor Purification

Cells were washed twice with PBS and detached with PBS containing 1 mM EDTA. The cells were collected by means of centrifugation for 10 min at 800 *g*, suspended in solubilization buffer containing 10 mM Tris-HCl (pH 7.4), 1% Triton X-100, 10% glycerol, 150 mM NaCl, 1 mM EDTA, 5 μg/ml leupeptin, 5 mM benzamidin, 1% aprotinin, 1 mM PMSF and incubated during 1 h on ice. The insoluble material was removed by centrifugation at 100,000 *g* for 1 h at 0°C. The clear supernatant was passed through a small column of Sepharose CL-4B (Pharmacia, Uppsala, Sweden) which contained covalently coupled 2E9, mAb directed against the external domain of the EGFR (11). The column was successively washed with 100 ml of the following buffers: (*a*) 10 mM Tris-HCl (pH 7.4), 0.1% Triton X-100, 10% glycerol, 1 M NaCl, 1 mM EDTA, 5 mM Benzamidin, 1% aprotinin, 1 mM PMSF; (*b*) 10 mM Tris-HCl pH (7.4), 10% glycerol, 1 mM EDTA, 5 mM benzamidin, 1% aprotinin, 1 mM PMSF; (*c*) 10 mM Lysine-NaOH (pH 9.5), 0.1% Triton X-100, 10% glycerol, 150 mM NaCl, 5 mM benzamidin; and (*d*) 10 mM Hepes (pH 5.0), 10% glycerol, 150 mM NaCl, 1 mM EDTA, 5 mM benzamidin. The bound EGF receptor was eluted from the affinity column with pH 11.5 elution buffer (10 mM Lysin-NaOH [pH 11.5], 0.05% Triton X-100, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 5 mM benzamidin). The eluate was immediately neutralized by the addition of 1 M Hepes (pH 7.4) and the purified receptor was stored at −80°C. The truncated receptor was from mouse NIH-3T3 fibroblasts transfected with EGF receptor cDNA of which the cytoplasmic domain was deleted (26), and was purified essentially as described above.

Binding of ^{125}I-Labeled EGF to the Purified Receptor

The ^{125}I-EGF was prepared by the chloramine-T method (10), specific activity varying between 200,000 and 900,000 cpm/ng EGF. Equilibrium EGF binding was performed in a final volume of 200 μl, which contained 10 ng of purified EGF receptor, 20 mM Hepes (pH 7.4), 0.1% BSA, and 0.1% Triton X-100. ^{125}I-EGF (0.5 ng/ml) and unlabeled EGF were mixed to a final EGF concentration varying from 0.1 to 200 ng/ml and binding was allowed for 2 h at room temperature. Nonspecific binding of these samples was determined separately by addition of 1,000 times excess of unlabeled EGF. Subsequently, the receptor complexes were precipitated by adding 0.5 ml 0.1% γ-globulin in 0.1 M phosphate buffer (pH 7.4) and 0.5 ml of 20.4% polyethylene glycol 6,000 at room temperature. The samples were mixed and centrifuged for 5 min at 14,000 *g*. The pellets were washed twice with 8.5% polyethylene glycol in 0.1 M phosphate buffer (pH 7.4). The radioactivity retained in the tubes was measured in a gamma counter (Crystal 5412 Multi Detector Ria system; United Technologies Packard, IL). The binding data were analyzed using the LIGAND program as described previously (3).

Protein Kinase Assay of the Purified EGFR

Autophosphorylation reactions were carried out in a final volume of 50 μl containing 20 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 3 mM $MnCl_2$, and 1 mM DTT. The EGFR (50 ng) was first incubated in this buffer with 100 ng/ml EGF for 20 min at 25°C and the kinase reaction was subsequently started by the addition of 10 μM (γ-^{32}P)ATP (10 μCi per assay) (Amersham International, Amersham, UK) and continued for 3 min at 30°C. The reaction was stopped by the addition of sample buffer (20 mM Tris-HCl [pH 6.8], 33% glycerol, 300 mM DTT, 6.7% SDS, and 0.01% bromophenol blue) and the samples were boiled for 3 min. The samples were analyzed on 10% SDS-PAGE and by autoradiography. The bands of the EGFR were excised from the gel and counted with a liquid scintillation counter.

Substrate phosphorylation was measured under the same conditions as described above using 5 mM angiotensin II as a substrate. The reaction was continued for 3 min at 30°C and stopped by the addition of 5 μl of 10 mg/ml BSA and 5 μl of 50% TCA. Proteins were precipitated by centrifugation and 40 μl of the supernatant was spotted onto P81 phosphocellulose filter (Whatman International, Maidstone, UK). The filters were washed four times with 7.5 mM phosphoric acid and counted in a liquid scintillation counter, and results are presented as the mean of three independent experiments.

Actin Purification

Actin was purified essentially as described before (13, 44) with minor modifications. A431 cells were washed twice with PBS and detached with PBS containing 1 mM EDTA. Cells were collected and pelleted at 800 *g* for 5 min and suspended in 2 vol of a buffer containing 20 mM Tris-maleate (pH 7.0), 1 mM EDTA, 0.5 mM ATP, 10 mM DTT, 0.34 M sucrose. The cells were lysed by Dounce homogenization on ice. The homogenate was clarified by centrifugation at 100,000 *g* for 1 h at 4°C. The clear supernatant was warmed to room temperature, supplemented with 75 mM KCl and 2 mM $MgCl_2$, and the solution was stirred for 90 min which induced polymerization of actin. The precipitate was collected by centrifugation at 14,000 *g* for 2 min and the pellet was suspended in ice-cold Tris-maleate (pH 7.0) containing 0.6 M KCl. After centrifugation for 3 h at 80,000 *g* the pellet was dissolved in buffer G containing 2 mM Tris-HCl (pH 8.0), 0.2 mM ATP, 0.5 mM β-mercaptoethanol, 0.2 mM $CaCl_2$, and 10% formamide. After dialysis against the same buffer the solution was centrifuged at 80,000 *g* for 3 h. The supernatant was mixed with DNAse I coupled to Sepharose CL-4B for 2 h at 4°C. After centrifugation at 2,000 *g* for 5 min the affinity matrix was intensively washed with buffer G, subsequently with buffer G containing 0.2 M NH_4Cl and again with buffer G. Elution was done with buffer G containing 40% formamide and the eluate was immediately diluted with buffer G without formamide to prevent denaturation of actin. The actin-containing fraction was concentrated by ultrafiltration using a Centrifugal Ultra Free unit (Millipore Corp., Bedford, MA) and stored in buffer G at 4°C.

SDS-PAGE and Immunoblotting

Protein determination was done using the BCA-reagent (Pierce Chemical Company, Rockford, IL) with BSA as standard. Proteins were solubilized in sample buffer (20 mM Tris-HCl [pH 6.8], 33% glycerol, 300 mM DTT, 6.7% SDS, and 0.01% bromophenol blue), separated on 10% SDS-PAGE either stained with Coomassie brilliant blue or blotted onto nitrocellulose (Schleicher and Schuell, Dassel, Germany) as described previously (39). The nitrocellulose was blocked with 0.3% milk powder (Nutricia, Zoetermeer, the Netherlands) in PBS for 45 min at room temperature. The filters were incubated with antibodies for 1 h diluted in 0.03% milk powder in PBS at room temperature. After washing in the same buffer the filters were 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 nitrobluetetrazolium salt and 0.17 mg/ml 5-bromo-4-chloro-3-indolyl phosphate toluidinium (Sigma Chemical Co.).

Electron Microscopy

Actin (300 μg/ml) was allowed to polymerize in vitro by the addition of $MgCl_2$ (final concentration 2 mM) and KCl (final concentration 75 mM). After 20 min of polymerization, grids covered with carbon-coated Formvar film were incubated for 30 s with the F-actin–containing solution. After a brief wash with polymerization buffer, the actin was negatively stained with 1% uranyl acetate. The grids were examined in a Philips CM10 electron microscope with an accelerating voltage of 100 kV.

Actin Sedimentation Assay

The binding of EGFR and synthetic peptides to F-actin was examined by the sedimentation assay. EGFR (2 μg), truncated EGFR (1 μg), six different proteins and synthetic peptides were incubated with 10 μg actin for 10 min in 200 μl buffer containing 2 mM Tris-HCl (pH 7.4), 0.2 mM $CaCl_2$, 0.2

mM DTT, and 0.5 mM ATP. Synthetic peptides HL-33 and HL-34 were prepared according to standard procedures and labeled with ^{125}I according to the chloramine-T method (10) yielding a specific activity of 150,000–200,000 cpm/μg peptide. For competition assays with the polyclonal anti-EGFR antibody 281-7, 100 ng EGFR was preincubated for 3 h with the antibody before adding the actin (10 μg). For competition assays with synthetic peptides, 100 ng EGFR was preincubated with 10 μg actin in the presence of 5 mg synthetic peptide. All competition experiments were performed in 200 μl buffer containing 2 mM Tris-HCl (pH 7.4), 0.2 mM $CaCl_2$, 0.2 mM DTT, and 0.5 mM ATP. In all cases polymerization of the actin was induced by adding 75 mM KCl and 2 mM $MgCl_2$ and after 1 h at room temperature the samples were centrifuged for 1 h at 100,000 *g*. The supernatants were removed, concentrated by ultrafiltration, and mixed with sample buffer. The pellets were immediately dissolved in sample buffer. Pellets and supernatants were either analyzed on 10% SDS-PAGE and stained with Coomassie brilliant blue or radioactivity in the pellet fraction was measured using a gamma counter. Nonspecific binding was determined in the presence of ~1,000-fold excess of unlabeled peptide and the results of two independent experiments are presented.

Results

EGFR and Actin Purification

The investigation of the direct interaction between the EGFR and actin in vitro, requires the purification of both proteins to homogeneity. The EGFR was purified from A431 epidermoid carcinoma cells, a well-known rich source of EGFR since this cell line has been reported to contain ~2 × 10^6 cell surface located receptors/cell, while these receptors represent ~20% of the total receptor population (8, 19). After cell extraction, the EGFR was isolated by affinity chromatography using the monoclonal anti-EGFR antibody 2E9 as a substrate. This antibody recognizes an extracellular peptide determinant of the EGFR (11). After elution of the affinity chromatography columns, the fractions were subjected to SDS-PAGE as described in Materials and Methods. As shown in Fig. 1 *A*, Coomassie brilliant blue staining of the purified fractions on gel revealed only one single protein band with an apparent molecular mass of 170 kD. Analysis of ^{125}I-EGF binding to this purified protein, according to the Scatchard method, revealed one single class of binding sites exhibiting an apparent dissociation constant K_D of 8.0 nM (Fig. 1 *B*).

To determine the kinase activity of the purified receptor, EGF (100 ng/ml) was added to the protein suspension in the presence of γ-^{32}P-ATP as described in Materials and Methods. SDS-PAGE, autoradiography, and subsequent quantification of incorporated radioactivity in the protein band revealed that EGF caused a twofold increase in receptor autophosphorylation, yielding a ratio of ^{32}P/receptor of ~1.5 (Fig. 1 *C*). In addition, the purified protein was able to phosphorylate exogenous substrates in the presence of EGF, as shown in Fig. 1 *D* for angiotensin II. In this particular experiment EGF caused a fourfold stimulation of angiotensin II phosphorylation within 3 min. These results demonstrate that the EGFR is purified to homogeneity, and exhibits EGF-binding capacity and EGF-induced tyrosine kinase activity.

Actin was also purified from A431 cells using a DNAse I affinity column as described in Materials and Methods. DNAse I has been shown to bind actin with a high affinity and specificity (44). After elution, SDS-PAGE and staining of the purified fractions by Coomassie brilliant blue, one single band was detected with an apparent molecular mass of 43 kD (Fig. 2 *A*). This purified protein is recognized by an antiactin antibody as shown by immunoblotting in Fig. 2 *B*. Under the conditions employed, actin is purified in its monomeric form, but is able to polymerize in vitro by the addition of $MgCl_2$ and KCl, as described by Zechel (44). The purified actin was incubated at room temperature for 20 min

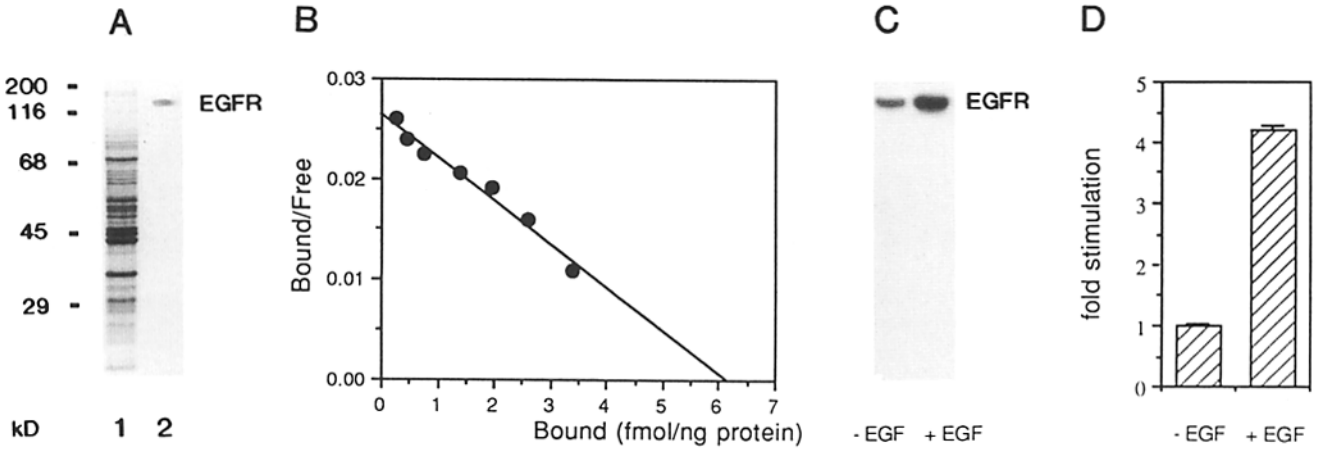


Figure 1. Purification and characterization of the EGFR. (*A*) The EGFR was purified from Triton X-100 extracts of A431 cells as described in Materials and Methods. The samples were analyzed on 10% SDS-PAGE and stained with Coomassie brilliant blue. (Lane *1*) Triton X-100 extract of A431 cells; (lane 2) purified EGFR. (*B*) Scatchard analysis of EGF binding to the purified EGFR. The receptor was incubated with different concentrations of EGF (0.1–200 ng/ml) for 2 h at room temperature and free EGF was removed by centrifugation for 5 min at 14,000 *g*. Binding data were analyzed using the LIGAND program. (*C*) Autophosphorylation of the purified receptor in the absence (−EGF) and presence (+EGF) of EGF. The receptor was preincubated with EGF for 20 min at 25°C and the kinase reactions were started by the addition of γ-^{32}P-ATP and continued for 3 min at 30°C. The reactions were stopped by the addition of sample buffer and the samples were boiled for 3 min. The samples were analyzed on 10% SDS-PAGE followed by autoradiography. (*D*) Angiotensin II phosphorylation by the purified EGFR in the absence (*−EGF*) and presence (*+EGF*) of EGF. Reactions were carried out as described above in the presence of 5 mM Angiotensin II and 50 ng purified EGFR. The reactions were stopped by the addition of 5 μl BSA (10 mg/ml) and 5 μl of 50% TCA. After centrifugation, 40 μl of the supernatant was spotted onto a P81 phosphocellulose filter washed four times with phosphoric acid and counted in a liquid scintillation counter.

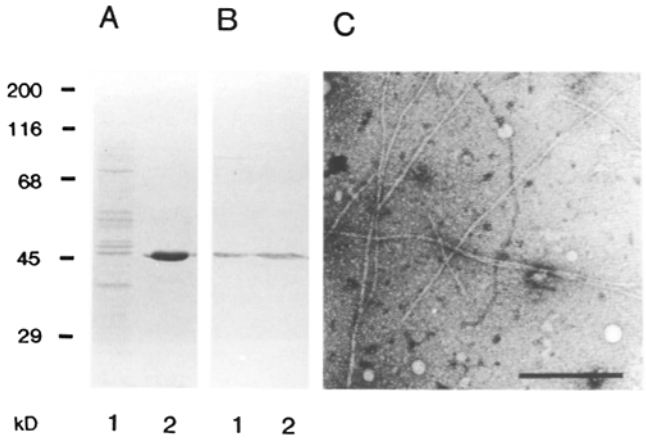


Figure 2. Purification and characterization of actin. (*A*) Actin was purified from A431 cells as described in Materials and Methods, analyzed on 10% SDS-PAGE, and stained with Coomassie brilliant blue. (Lane *1*) Supernatant of A431 cell lysates after Dounce homogenization and centrifugation; (lane *2*) purified actin after DNAse I affinity chromatography. (*B*) Immunoblot analysis of the purified actin. A431 cell lysates and purified actin were separated on 10% SDS-PAGE, blotted onto nitrocellulose, and analyzed for the presence of actin using a monoclonal antiactin (Amersham International, Amersham, UK). (Lane *1*) A431 cell lysates; (lane *2*) purified actin. (*C*) Electron micrograph of the purified actin. Actin was allowed to polymerize in vitro by the addition of $MgCl_2$ and KCl. Samples of the polymerized actin were attached to grids covered with carbon-coated formvar film, and negatively stained with 1% uranyl acetate. Bar, 0.1 μm.

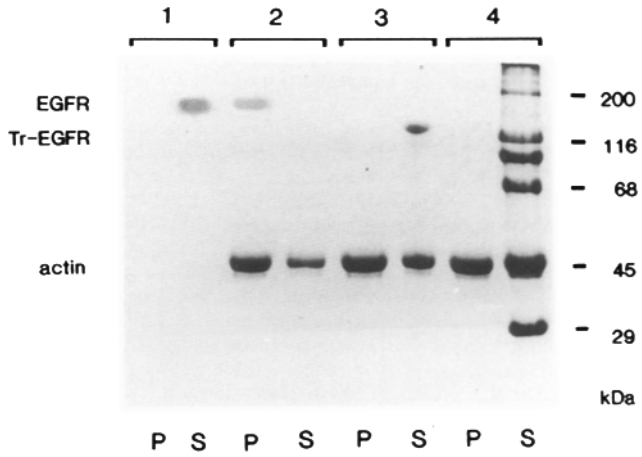


Figure 3. Actin sedimentation in the presence of EGFRs and control proteins. The EGFR, the truncated EGFR, G-actin, and six different control proteins were incubated as indicated. G-actin was allowed to polymerize for 1 h at room temperature, and after centrifugation the pellets (*P*) and supernatants (*S*) were analyzed on 10% SDS-PAGE and stained with Coomassie brilliant blue. (Lanes *1*) EGFR (2 μg); (Lanes *2*) EGFR (2 μg) and G-actin (10 μg); (Lanes *3*) truncated EGFR (1 μg) and G-actin (10 μg); (Lanes *4*) control proteins and G-actin (10 μg).

in the presence of 10 mM $MgCl_2$ and 75 mM KCl. A sample of the F-actin containing solution was prepared for electron microscopical examination as described in Materials and Methods. As shown in Fig. 2 *C*, the purified protein is able to polymerize to filamentous structures having a diameter of ~4 nm, which is in agreement with the diameter of actin microfilaments (25).

Association of the EGFR with Actin In Vitro

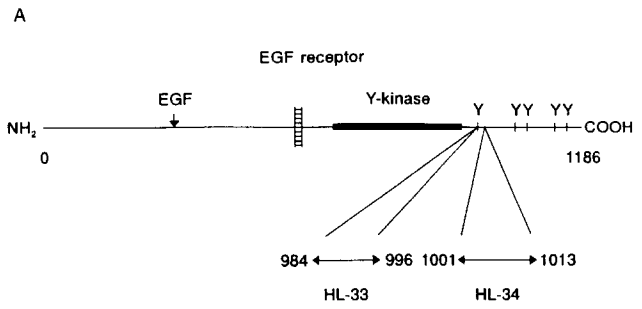
Aliquots of purified EGFR and of purified actin were incubated at room temperature in the presence of $MgCl_2$ and KCl for 60 min as indicated. Subsequently the actin filaments were sedimented by centrifugation at 100,000 *g* for 1 h at room temperature. The supernatant containing unpolymerized actin was removed and concentrated by ultrafiltration. Samples of both pellet and supernatant were separated on SDS-PAGE and analyzed after Coomassie brilliant blue staining. In the absence of actin, the EGFR was detected in the supernatant while no EGFR was present in the pellet fractions (Fig. 3, lanes *1P* and *S*). However, in the presence of actin and after actin polymerization, the EGFR was found to be present in the pellet and not in the supernatant (Fig. 3, lanes *2P* and *S*). These results demonstrate that all EGFRs bind to F-actin, but not to G-actin, since the supernatant fraction (Fig. 3, lane *2S*) did contain actin but no EGFR.

To determine the specificity of the association of the EGFR to F-actin, a number of control experiments were performed. A truncated EGFR was purified from NIH-3T3 cells expressing an EGFR that lacks almost the entire cytoplasmic domain. Previously we have shown that the EGFR of this mutant is not associated with the cytoskeleton in vivo (35). Incubation of the purified truncated EGFR in the presence of actin and subsequent actin polymerization, followed by analysis on SDS-PAGE as described above, reveals that the truncated receptor remains in the supernatant and not in the pellet (Fig. 3, lanes *3P* and *S*), in contrast to the wild type EGFR (Fig. 3, lanes *2P* and *S*). Furthermore, in order to exclude a possible nonspecific association of the receptor to F-actin by trapping of the receptor in the actin filament network, the same experiment was performed using a mixture of proteins with molecular masses of 200, 116, 98, 68, 45, and 29 kD, respectively. As shown in Fig. 3 (lanes *4P* and *S*), none of these proteins were found in the pellet, but instead they were present in the supernatant.

These results clearly demonstrate that the association of the EGFR to F-actin is highly specific and requires the cytoplasmic domain of the receptor. Furthermore, it is shown that in vitro the EGFR binds directly to F-actin with no other proteins involved, demonstrating that the EGFR is an actin-binding protein.

Effect of Antibody 281-7 on EGFR Binding to F-Actin

Of particular interest appears the observation that the amino acid sequence of the EGFR domain involving amino acid residues 986–999 appears to have high homology with the actin-binding domain of profilin of *Acanthamoeba* (39) (Fig. 4). To obtain evidence on the involvement of this domain of the EGFR in the binding to actin, we have determined the effect of the polyclonal anti EGFR antibody 281-7 on the cosedimentation of the EGFR with actin. This antibody was raised against a synthetic peptide corresponding to amino acid residues 984–996 (HL-33) of the EGFR (11) (Fig. 4). A preincubation of the antibody 281-7 and the EGFR, followed by incubation with actin, subsequent actin polymerization, and detection on SDS-PAGE of both supernatant and pellet fractions revealed that preincubation of the antibody and EGFR prevented the interaction of the EGFR with actin.



HL-33	984						990						996
EGF receptor	Asp	Asp	Val	Val	Asp	Ala	Asp	Glu	Tyr	Leu	Ile	Pro	Gln
Acanthamoeba	112									120			123
profilin	Val	Val	Glu	Lys	Leu	Ala	Asp		Tyr	Leu	Ile	Gly	Gln
HL-34	1001									1010			1013
EGF receptor	Ser	Ser	Pro	Ser	Thr	Ser	Arg	Thr	Pro	Leu	Leu	Ser	Ser

Figure 4. Characterization of synthetic peptides. (*A*) Schematic representation of the EGFR. Indicated are the EGF-binding site, the transmembrane region, the tyrosine kinase domain (*Y-kinase*) and the autophosphorylation sites (*Y*). Synthetic peptides HL-33 and HL-34 are homologous with the indicated regions of the cytoplasmic domain of the receptor. (*B*) Sequence comparison of HL-33 and HL-34 with *Acanthamoeba* profilin. Residues of HL-33 that are homologous with profilin are indicated and this sequence has been suggested as being the actin-binding site (33).

Under these conditions the EGFR and antibody 281-7 were found to be present in the supernatant and not in the pellet fractions (Fig. 5, lanes *P* and *S*), which is clearly in contrast to EGFR incubated in the absence of antibody 281-7 (Fig. 3, lanes *2P* and *S*). These results indicate that the antibody and F-actin compete for binding to the EGFR. This competition may be due to steric hindrance, in that the antibody is masking the actin-binding site of the EGFR. Alternatively, the competition may be caused by the fact that antibody 281-7 and F-actin bind to the same domain of the EGFR.

Identification of the Actin-binding Domain of the EGFR

To determine whether the amino acid residues 984–996 represent the actin-binding domain of the EGFR, synthetic peptide HL-33 was analyzed for actin-binding capacity using the sedimentation assay. As a control, a synthetic peptide homologous to residues 1001–1013 (HL-34) was used (Fig. 4). Both peptides, HL-33 and HL-34, were iodinated as described in Materials and Methods and the ^{125}I-labeled peptides were incubated in the presence of actin. After actin polymerization, the F-actin was separated from the unpolymerized actin by centrifugation, and the radioactivity was determined in pellet and supernatant fractions. As shown in Fig. 6 *A*, HL-33 binds to F-actin in contrast to peptide HL-34. The specificity of peptide binding was analyzed by measuring peptide binding in the presence of excess unlabeled peptide (Fig. 6 *A*). These results demonstrate unequivocally that the cytoplasmic domain of the EGFR containing amino acid residues 984–996 is able to bind directly to F-actin, with no other proteins involved.

An important question is whether the residues 984–996 represent the only actin-binding site of the EGF receptor. To test this, the sedimentation assay was performed in the presence of either HL-33 or HL-34. As shown in Fig. 6 *B*, HL-33 competes for actin binding of the EGFR while HL-34 does not, demonstrating that residues 984–996 of the cytoplasmic domain of the EGFR represents the only actin-binding site of the EGFR.

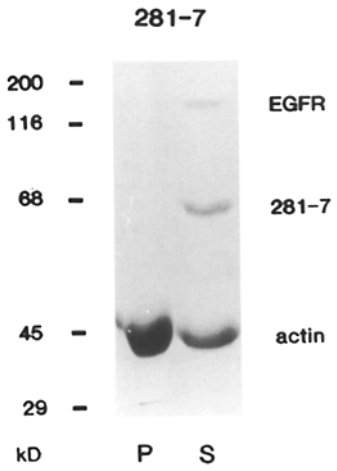


Figure 5. Effect of antibody 281-7 on EGFR cosedimentation with actin. EGFR (100 ng) was preincubated in the presence of anti-EGFR antibody 281-7 as described in Materials and Methods. After adding 10 μg actin, polymerization of actin was performed for 1 h at room temperature. After sedimentation the pellet (*P*) and supernatant (*S*) were analyzed on 10% SDS-PAGE and stained with Coomassie brilliant blue.

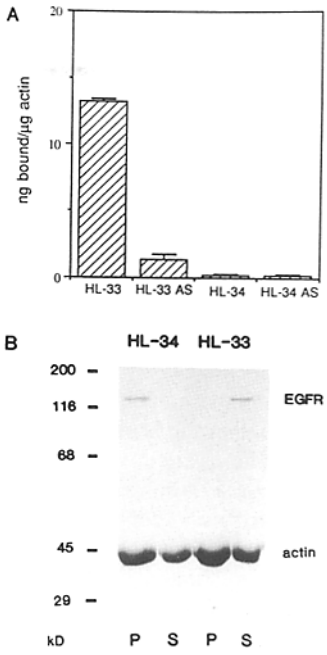


Figure 6. Analysis with synthetic peptides HL-33 and HL-34. (*A*) Binding of HL-33 and HL-34 to actin. Synthetic peptides were labeled with ^{125}I. Actin (30 μg) was incubated with HL-33 (1 μg) and HL-34 (1 μg). After polymerization, actin was precipitated by centrifugation for 1 h at 100,000 *g*. Binding of the synthetic peptides to the actin pellet (*HL-33*, *HL-34*) was measured in a gamma counter. Nonspecific binding was determined by 1,000 times excess nonlabeled peptide (*HL-33 AS*, *HL-34 AS*). (*B*) Effect of synthetic peptides HL-34 and HL-33 on EGFR cosedimentation with actin. Actin (10 μg) was incubated with the EGFR (100 ng) in the presence of 5 mg synthetic peptides HL-34 and HL-33. Actin was allowed to polymerize for 1 h at room temperature. After sedimentation the pellets (*P*) and supernatants (*S*) were analyzed on 10% SDS-PAGE and stained with Coomassie brilliant blue.

Discussion

A number of recent studies have shown that growth factor receptors may be associated in vivo, to the cytoskeleton, including receptors for nerve growth factor (34), PDGF (45), and EGF (41). Especially the interaction of the EGFR with the cytoskeleton has gained considerable interest, since binding studies demonstrated that the cytoskeleton-associated EGFR was of the high affinity class (37, 41), and this high-

affinity class was shown to be responsible for EGF-induced signal transduction (2, 11). An interesting aspect of the interaction between the EGFR and the cytoskeleton involves the nature of the cytoskeletal component. As judged by the diameter of the elements as observed in the electron microscope, it has been suggested that actin is involved in the association (41). This suggestion was supported by a colocalization of EGFR and actin filaments using immunofluorescence microscopy (5, 28, 38). Furthermore, it was shown that immunoprecipitation of the cytoskeleton-associated EGFR resulted in a coprecipitation of actin (28), while selective extraction of cytoskeletons revealed also an interaction between EGFR and actin filaments (38).

Although these results clearly demonstrate that the EGFR is bound to actin, it is not known whether the EGFR is bound directly to actin or whether other proteins are involved in this association. Therefore, we have studied in this paper the interaction of EGFR and actin in vitro using highly purified components. EGFR and actin were purified and they were shown to be biologically active. The isolated EGFR binds EGF with a K_D of 8.0 nM and EGF binding stimulates tyrosine kinase activity and actin is shown to polymerize in vitro into 4-nm filaments. Using the cosedimentation assay, it is demonstrated that all EGFRs are able to bind directly to actin via its cytoplasmic domain with no other proteins involved. The actin-binding domain of the EGFR is present in residues 984–996 and appears to be the only actin-binding site of the EGFR. These findings are supported by the homology of the EGF receptor domain containing amino acid residues 989–994 with that of the actin binding site of profilin of *Acanthamoeba* (35).

As shown in this paper, all EGFRs are able to bind to actin in vitro which is in agreement with the fact that no heterogeneity exists in the EGF-receptor primary structure (33). In vivo, however, only 5% of the receptor population appears to be associated to the cytoskeleton (37, 41) which suggest that in vivo the binding of EGFR to actin filaments is subject to stringent regulation. An explanation for this observation is a possible competition for the actin-binding site of the EGFR between actin and other proteins. Alternatively, regulation of actin polymerization may be involved in modulating EGFR association to the cytoskeleton. EGF is stimulating actin polymerization in the cortical skeleton while at the same time an increased number of cytoskeleton-associated EGFRs is observed (28, 37). A possible regulatory way for actin polymerization involves the action of profilin, a well known actin-binding protein. The activity of profilin is altered upon association with $PtdInsP_2$ (25, 43). Binding of profilin to $PtdInsP_2$ results in dissociation of profilin-actin complexes, resulting in actin polymerization (20). Furthermore, activation of the EGFR tyrosine kinase by binding of EGF results in activation of several kinases involved in the conversion of PtdIns to $PtdInsP_2$ (23, 40). The increased level of $PtdInsP_2$ may result in an increased binding of profilin and subsequent actin polymerization. Furthermore, the increased F-actin content may then in its turn bind to the EGFR, thereby explaining the observed EGF-induced association of EGFR to the cytoskeleton (37). In addition, the regulation of actin polymerization may be influenced by other actin-binding proteins which may be regulated by EGF-induced activation of the receptor kinase.

An intriguing aspect concerns the biological function of the interaction of EGFR with actin. The cytoskeleton-associated EGFRs appear to be high affinity receptors, which suggests that the cytoskeleton is involved in the determination of receptor affinity (5). However, EGF induces the association of low affinity receptors to the cytoskeleton, indicating that the cytoskeleton alone is unlikely to represent the primary determinant of receptor affinity (37). Alternatively, it has recently been demonstrated that a number of proteins involved in EGF-induced signal transduction, such as PtdIns-4 kinase, PtdIns(4)P-5 kinase, diacylglycerol kinase, and phospholipase C, are also associated to the actin microfilament system (24). These findings suggest that the actin microfilaments may act as a matrix in the formation of protein complexes consisting of the receptor and its substrates. This matrix may increase the efficiency of signal transduction between the receptor and its substrates. Support for this hypothesis has been obtained by Rebillard and coworkers who showed that the actin microfilament system is involved in regulation of oncogene expression (27). Furthermore, translocation of activated protein kinase C was found to depend upon actin filaments (17). These findings suggest that the actin microfilaments are involved in gene regulation either by interacting directly with the signal transduction cascade, or by compartmentalization of regulatory molecules in responses to signals arriving at the cell surface. By regulating the actin microfilament system, EGF may regulate the cellular system for optimal signal transduction.

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