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) 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.
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. EGFR 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 cm² roller bottles (Falcon, Becton Dickinson Labware, UK) to confluence.

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 and 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 g/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 10,000 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 completely coupled 219, 386 g of the external domain of the EGFR (13). 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 Lysine-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 125I-Labeled EGF to the Purified Receptor
The 125I-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 ml, which contained 10 ng of purified EGF receptor, 20 mM Hepes (pH 7.4), 0.1% BSA, and 0.1% Triton X-100. 125I-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 5 ml of 20% polyethylene glycol 6000 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).
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, EGFR (100 ng/ml) was added to the protein suspension in the presence of $^{32}$P-ATP as described in Materials and Methods. SDS-PAGE, autoradiography, and subsequent quantification of incorporated radioactivity in the protein band revealed that EGFR 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 antiantibody 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](image_url)

*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 J) 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 $^{32}$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.*
in the presence of 10 mM MgCl₂ 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 into 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 MgCl₂ 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 supernatants but not in the pellet. In the presence of actin, the EGFR was found to be present in the pellet and not in the supernatant. 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).

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. 3). 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.

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 kDa, 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.
The EGFR (ErbB-1) and other related EGFR family members have many functions in cell biology, including mitogenic stimulation and the regulation of cell growth, differentiation, and apoptosis. These receptors contain an extracellular ligand-binding domain, a transmembrane region, and an intracellular tyrosine kinase domain. The tyrosine kinase domain is activated by autophosphorylation and can phosphorylate other proteins, leading to cellular responses.

The extracellular domain of the EGFR binds to the ligand EGF (epidermal growth factor) and other growth factors, such as TGF-α (transforming growth factor-α), which is similar to EGF. This binding triggers the activation of the receptor kinase, leading to downstream signaling events.

Steric hindrance can play a role in the ability of EGFR to bind to F-actin. In the presence of antibody 281-7, EGFR and F-actin bind to the same domain of the EGFR, with antibody 281-7 possibly masking the actin-binding site of the EGFR. Alternatively, residues of HL-33 that are homologous with profilin are indicated and this sequence has been suggested as being the actin-binding site.

To determine whether the amino acid residues 984–996 represent the actin-binding domain of the EGFR, synthetic peptides HL-33 and HL-34 were iodinated and labeled with 125I. Actin (10 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).

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 radioactivity was determined in a gamma counter.

An important question is whether the residues 984–996 represent the only 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.

Discussion

A number of recent studies have shown that growth factor receptors may be associated with the cytoskeleton, including receptors for nerve growth factor (34), PDGF (45), and EGF (41). 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 interaction is important for cell signaling.
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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