Identification of an intracellular domain of the EGF receptor required for high-affinity binding of EGF

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Abstract Although all EGF receptors in EGF receptor-expressing cells are molecularly identical, they can be subdivided in two different classes that have either a high or a low affinity for EGF. Specifically the high-affinity class is associated with filamentous actin. To determine whether the interaction of the EGF receptor with actin induces its high-affinity state, we studied EGF-binding properties of an EGF receptor mutant that lacks the actin-binding site. Interestingly, we found that cells expressing this mutant receptor still display both high- and low-affinity classes of EGF receptors, indicating that the actin-binding domain does not determine the high-affinity binding state. By further mutational analysis we identified a receptor domain, within the tyrosine kinase domain, that regulates the affinity for EGF.

Key words: EGF; EGF receptor; Scatchard analysis; Cytoskeleton; F-actin

1. Introduction

The epidermal growth factor stimulates proliferation and differentiation in a variety of cell types [1,2]. The EGF receptor is a transmembrane tyrosine kinase that dimerizes upon ligand binding [3,4]. Receptor dimerization leads to activation of the EGF receptor tyrosine kinase and subsequently to crossphosphorylation of the receptor [1,2]. Analysis of EGF binding to a great variety of cells has revealed that EGF is bound with different affinities. This phenomenon is usually interpreted by the presence of two subpopulations of EGF receptors with different affinities, a high-affinity class with an apparent equilibrium dissociation constant (K_d) of 0.01–0.2 nM and a low-affinity class with an apparent K_d of 2–20 nM [5,6]. The existence of two subpopulations of EGF receptors is also suggested by the observation that different monoclonal antibodies specifically block EGF-binding to either the low or the high-affinity class [7,8]. Using these antibodies it has been shown that the high-affinity class is characterized by a fast association rate and a slow dissociation rate (fast/slow), while the low-affinity receptors are either of the fast/fast or slow/slow type [9]. In addition, these antibodies were used to demonstrate that the high-affinity receptor class is required and sufficient for all EGF-induced responses [7,8].

The molecular background for the difference in affinity of EGF receptors is still unclear. This question is especially intriguing considering the fact that both receptor classes originate from the same primary sequence. It has been suggested that the high-affinity form of the EGF receptor is the result of receptor dimerization [3,4,10–12]. The increase in affinity of receptor dimers could be explained by a reduced dissociation rate of EGF [13]. However, dimerization does not readily explain the increased association rate of EGF to high-affinity receptors as reported by Berkers et al [9]. An alternative explanation is that the intracellular domain of the EGF receptor regulates its affinity for EGF. The first indication for this notion was the observation that activation of PKC by phorbol esters converted the high-affinity form into a low-affinity form [5]. Moreover, cells expressing EGF receptors lacking the entire intracellular domain posses only low-affinity receptors [14,15]. It is, however, unknown which part of the intracellular domain of the EGF receptor is required for the induction of the high-affinity state of the receptor. Deletion of the major autophosphorylation sites or PKC phosphorylation sites did not alter the affinity of the receptor [16,17]. Moreover, inactivation of the tyrosine kinase by a substitution of the lysine at position 721 to phenylalanine did not affect the affinity of the receptor [18,19].

We and others have demonstrated that specifically the high-affinity class of EGF receptors is associated with the cytoskeleton [20–24]. By using a co-sedimentation assay with both purified EGF receptors and actin, we have previously found that the EGF receptor binds directly to actin [25]. The actin-binding domain (ABD) of the EGF receptor was shown to be located between amino acids 984 and 996 [25]. These data suggest that the binding of EGF receptors to actin could be involved in the formation of the high-affinity state. In this study we investigated whether the direct binding of the EGF receptor to actin induces the high-affinity state of the EGF receptor. Our data show that this is not the case. In stead, another domain of the intracellular part of the receptor, located within the tyrosine kinase domain, regulates the affinity for EGF.

2. Materials and methods

2.1. EGF receptor cDNA constructs

EGF receptor cDNA from the pSV2HERc vector was used to make mutant EGF receptors [26]. The Δ134 deletion was created by ligating the *Cla*I site at position 3007 with the compatible *Nar*I site at position 3409, resulting in a deletion from amino acids 918 to 1052. For the T963 mutant, the EGF receptor cDNA was truncated at the *Nsi*I site at position 3145. The Δ19 deletion was created by ligating the *Bcl*I site at bp 3020 with the *Bcl*I site at bp 3077, resulting in a deletion from amino acids 921 to 940. In order to construct the Δ12 ABD deletion the 618-bp *Hinc*II–*Cla*I fragment was placed in a pBluescript-II-SK$^-$ vector (Stratagene, La Jolla, CA, USA). From this construct a 640-bp *Hin*dIII–*Kpn*I fragment was isolated and ligated in a pALTER-1 vector (Promega, Madison, WI, USA). Site-directed mutagenesis was performed using the primer 5′-CTGCTGAAGAAGCCCTGGTCCATGTCTTCTTCATCC-3′, according to procedures of the manufacturer, resulting in a deletion from amino acids 984 to 996. All

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constructs were checked by dideoxy-sequencing, and mutated EGF receptor cDNA constructs were placed into the expression vector pSV_2 under the control of the SV40 promoter.

2.2. Transfections and tissue culture

Mouse NIH 3T3 (clone 2.2) cells, expressing no detectable amounts of murine EGF receptors [18], were seeded 5 h prior to transfection at a density of $1.3x10^4$ per cm^2. Cells were co-transfected with 30 μg EGF receptor construct and 10 μg pSV_2NEO using a modified calcium-phosphate precipitation technique at a CO_2 concentration of 4% [27]. 20 h after transfection the medium was re-freshed and CO_2 concentration was re-adjusted to 7%. The next day cells were split and seeded at a density of $2.5\times10^2/cm^2$ and put under G418 resistance selection using 550 μg/ml G418 (Sigma, St. Louis, MO, USA). Resistant clones were picked up after 2 to 3 weeks and screened for ^{125}I-EGF binding or EGF receptor expression. NIH 3T3 fibroblasts transfected with the different EGF receptor constructs were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, UK) supplemented with 7.5% foetal calf serum (Gibco) and 250 μg/ml G418 at 37°C in a humidified atmosphere containing 7% CO_2.

2.3. Actin purification and actin co-sedimentation assay

Rabbit skeletal muscle actin was purified as described by Pardee and Spudich [28] with minor modifications and stored as acetone powder at −20°C. EGF receptors were purified by affinity chromatography using EGF-coupled Sepharose CL-4B (Pharmacia, Uppsala, Sweden) as described before [25]. Binding of purified EGF receptor to purified actin was examined by the actin-co-sedimentation assay as described earlier [25] and the different fractions were analyzed by Western blotting using an anti-EGF receptor antibody (03-5600, Zymed Lab., San Francisco, CA, USA).

2.4. EGF-binding experiments

EGF was iodinated using the chloramine-T method yielding specific activities of 500 000–900 000 cpm/ng EGF (EGF receptor grade; Collaborative Research, Waltham, MA; ^{125}I, Amersham International, UK). EGF-binding experiments were all performed at 4°C and cells were incubated with the different concentrations of EGF for at least 3 h. During this time interval EGF-binding has reached equilibrium as has been decribed previously [9]. Non-specific binding was determined using 1000-fold excess of unlabeled EGF. The binding data were analyzed according to Scatchard using the LIGAND program as previously described in detail [9].

3. Results

To test whether the actin-binding site is important for the formation of the high-affinity EGF receptor we constructed a mutant EGF receptor lacking the actin-binding domain. The ABD of the EGF receptor is located between residues 984 and 996 and these amino acids were removed by an in-frame deletion (Fig. 1). This construct, designated as Δ12, was stably expressed in mouse NIH 3T3 fibroblasts lacking detectable levels of endogenous EGF receptors. In order to confirm that this ABD sequence is essential for direct binding of the

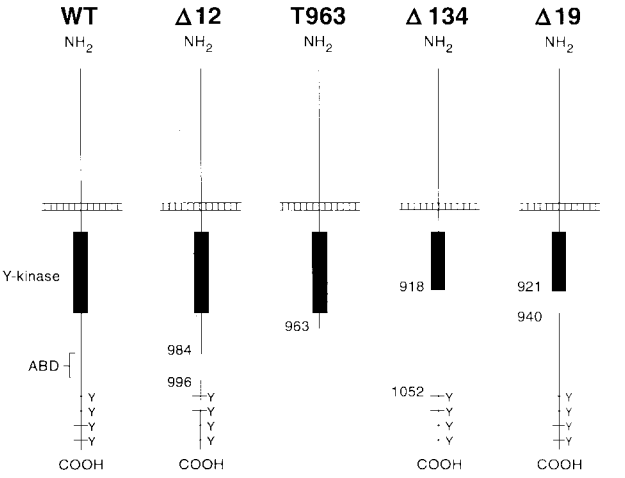

Fig. 1. Schematic representation of wild-type and mutant EGF receptors. Y-kinase, tyrosine kinase domain; ABD, actin-binding domain; Y, tyrosine autophosphorylation site; WT, wild-type EGF receptor; Δ12, EGF receptor lacking the ABD; T963, EGF receptor truncated at residue 963; Δ134, EGF receptor lacking amino acids 918–1052; Δ19, EGF receptor lacking amino acids 921–940.

EGF receptor to actin, wild-type and Δ12 receptors were isolated from NIH 3T3 cells by affinity chromatography. Actin was purified from rabbit skeletal muscle as described previously [8]. Binding of wild-type and mutant EGF receptors to actin was measured by an actin-co-sedimentation assay. In this experiment, the purified components were mixed and actin polymerization was induced by adding KCl and $MgCl_2$. Filamentous actin was separated from non-polymerized actin by centrifugation, and pellet and supernatant fractions were analyzed for the presence of EGF receptors by Western blotting. As shown in Fig. 2, wild-type receptors co-sedimented with F-actin and are found in the pellet (P) fraction, while the Δ12 receptors remained in the supernatant (S). In the absence of actin, both EGF receptors remained quantitatively in the supernatant fraction. These data show that the Δ12 mutant receptors do not bind to purified F-actin, which confirms our previous data that these 12 amino acids constitute the actin-binding domain [25].

Subsequently, we determined EGF binding to NIH 3T3 fibroblasts expressing Δ12 EGF receptors. Control and Δ12 cells with similar receptor numbers were incubated with a fixed concentration of ^{125}I-EGF (0.5 ng/ml) and increasing amounts of non-labeled EGF (0.5–500 ng/ml). The amount of radiolabeled EGF bound to the cells was determined and the data were analyzed according to Scatchard as described

Table 1
Equilibrium binding parameters for wild-type EGF receptor and EGF receptor mutants

Cell line	$K_{d,1}$ (nM)	N_1 ($\times10^3$/cell)	$K_{d,2}$ (nM)	N_2 ($\times10^3$/cell)
HERc13	0.07 ± 0.02	11.3 ± 4.1	12.6 ± 4.1	71.8 ± 13.6
Δ12–21	0.09 ± 0.01	15.9 ± 4.6	4.3 ± 2.2	81.2 ± 32.5
T963	0.19 ± 0.09	33.6 ± 13.0	10.8 ± 4.9	82.3 ± 11.1
Δ134	–	–	3.9 ± 1.2	5.4 ± 0.8
HERc9	0.08 ± 0.01	1.6 ± 0.2	9.0 ± 0.1	17.7 ± 0.8
Δ19	–	–	13.3 ± 2.2	21.2 ± 0.8

Subconfluent monolayers of NIH 3T3 cells expressing either wild-type or mutated EGF receptors were incubated with a fixed amount (0.5 ng/ml) of ^{125}I-EGF and increasing amounts (0.5–500 ng/ml) of non-labeled EGF at 4°C for 3 h. The binding data were analyzed according to the method of Scatchard. $K_{D,1}$ and $K_{D,2}$ are the apparent dissociation constants of the high- and low-affinity binding sites, respectively; N_1 and N_2 are receptor numbers per cell of high- and low-affinity binding sites, respectively. Each value represents the mean result of at least three independent binding experiments each performed in triplicate. The S.E.M. of the individual binding data is indicated.

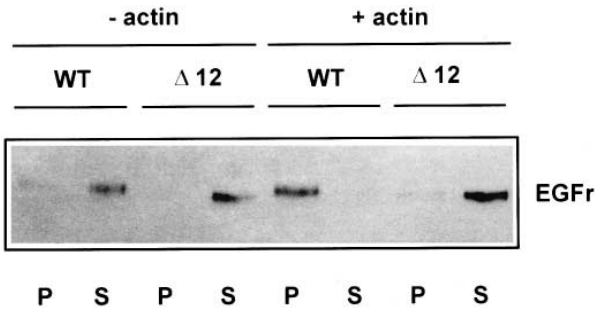

Fig. 2. Cosedimentation of F-actin with wild-type and Δ12 EGF receptors. Purified wild-type (WT) or Δ12 EGF receptors were pre-incubated with buffer alone (−actin) or purified G-actin (+actin). Actin polymerization was started by adding 75 mM KCl and 2 mM MgCl2 and polymerization was continued for 1 h at room temperature. Subsequently, F-actin and G-actin were separated by centrifugation for 1 h at 100 000 × *g* and both pellet (P) and supernatant (S) fractions were analyzed for the presence of EGF receptors by Western blotting.

previously [9]. A curvi-linear Scatchard plot was found for both cell types, demonstrating the presence of two affinity classes both in the wild-type and in the Δ12 cells. The high-affinity class of the wild-type EGF receptors had an apparent K_d of 0.07 ± 0.02 nM and the low-affinity class an apparent K_d of 12.6 ± 4.1 nM (Table 1). The equilibrium dissociation constants obtained from the Δ12 receptors were 0.09 ± 0.01 nM and 4.3 ± 2.2 nM, respectively. To exclude possible clonal differences we analyzed several other clones expressing different receptor numbers of wild-type or mutant receptors and these showed similar results (data not shown). In conclusion, these experiments demonstrate that the direct binding of the EGF receptor to actin is not responsible for the formation of high-affinity EGF receptors.

As shown previously, NIH 3T3 cells expressing EGF receptors lacking the entire intracellular region, bind EGF with low affinity [14,15]. In order to map the specific domain of the EGF receptor that is required for high-affinity binding we analyzed EGF binding to NIH 3T3 cells expressing different truncated or deletion mutant receptors. One of such mutant receptors was truncated at amino acid 963 (T963); another lacked 134 amino acids between positions 918 and 1052 (Δ134) (Fig. 1). Analysis of EGF binding revealed a curvi-linear Scatchard plot for the T963 truncation mutant but a linear plot for the Δ134 in-frame deletion mutant. Two affinity classes with apparent K_d values of 0.19 ± 0.09 nM and 10.8 ± 4.9 nM, respectively, were present in T963 cells, but only the low-affinity class (apparent K_d of 3.9 ± 1.2 nM) was present in Δ134 cells. This suggests that an EGF receptor domain responsible for high-affinity EGF binding had been lost by the Δ134 deletion. Comparison of the remaining sequences of the T963 mutant that were deleted in the Δ134 mutant (Fig. 1) predicts that the sequence from amino acids 918 to 963 contains a sequence responsible for high-affinity binding of EGF.

To test this hypothesis we constructed an additional EGF receptor mutant which contained the ABD but had a deletion from amino acids 921 to 940 (Δ19) (Fig. 1). Binding data of this mutant revealed a linear Scatchard plot indicating the presence of only the low-affinity class with an apparent K_d of 13.3 ± 2.2 nM (Table 1). Expression of the Δ134 and Δ19 mutant receptors was very low, a phenomenon observed before for certain EGF receptor mutants [15]. Scatchard analysis of cells expressing a similar number of wild-type EGF receptors (HERc9 cells) showed the presence of two affinity classes indicating that the absence of high-affinity receptors is not simply due to the low receptor number (Table 1). In conclusion, these results indicate that the EGF receptor domain from amino acids 921 to 940 is required for the induction of the high-affinity class of EGF receptors.

4. Discussion

In this paper we investigated whether the direct binding of EGF receptors to filamentous actin is involved in the formation of high-affinity EGF receptors. We generated a mutant EGF receptor that lacked the entire actin-binding domain and as a consequence did not bind to filamentous actin. After expression of this receptor in NIH 3T3 cells we could still observe the presence of both high- and low-affinity receptors. This demonstrates that actin association of the EGF receptor is not involved in the formation of the high-affinity state. Subsequently, we analyzed EGF binding to NIH 3T3 cells expressing several EGF receptor mutants and we were able to define a 19-amino-acid domain of the intracellular part of the EGF receptor that is required for the acquisition of high-affinity receptors. The 19-amino-acid domain is located within the tyrosine kinase domain in a region that has been designated as subdomain XI [29]. This subdomain has been implicated in the binding of peptide substrates and initiation of phosphotransfer [29].

An interesting question is how subdomain XI might affect the affinity of the receptor for EGF. A trivial explanation would be that the deletion of 19 amino acids results in a conformational change of the EGF-binding site. This seems unlikely since similar deletion mutations, e.g., the Δ12 mutant, do not have such an effect. Another possibility, based on the idea that formation of the high-affinity state is induced by receptor dimerization, would be that subdomain XI is involved in EGF receptor dimerization. However, also this explanation seems unlikely since recent data have demonstrated that receptor dimerization is regulated either by the ligand or by the extracellular domain of the receptor [30]. Furthermore, mutant EGF receptors lacking the intracellular domain are still able to dimerize with intact EGF receptors [31].

An alternative explanation may be that subdomain XI binds to an affinity-modulating protein. Evidence for the existence of these kind of proteins comes from the work of Walker and Burgess [32]. They have found that, when plasma membrane vesicles isolated from EGF receptor-expressing cells are pre-treated with either PDGF or EGF, the high-affinity receptors are transmodulated into low-affinity receptors. It was shown that, during this pre-treatment, several phosphorylated proteins were released from the vesicles. Interestingly, when the released proteins were dephosphorylated in vitro and added back to the transmodulated membrane vesicles the high-affinity state of the receptor was restored completely [32]. These findings indicate that these proteins can act as affinity-modulating factors and that their action is regulated by their phosphorylation state. In this light, it is tempting to suggest that an affinity-modulating protein binds to subdomain XI within the receptor tyrosine kinase domain and is a substrate of this kinase. Current research is aimed at

the identification of this putative subdomain XI-binding factor.

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