Journal of Cellular Biochemistry 39:455–465 (1989)
Growth Factors and Their Receptors: Genetic
Control and Rational Application 207–217

Ligand-Induced Association of Epidermal Growth Factor Receptor to the Cytoskeleton of A431 Cells

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Recently, we have obtained evidence in favor of a structural interaction between the epidermal growth factor (EGF) receptor and the Triton X-100-insoluble cytoskeleton of epidermoid carcinoma A431 cells. Here we present a further analysis of the properties of EGF receptors attached to the cytoskeleton. Steady-state EGF binding studies, analyzed according to the Scatchard method, showed that A431 cells contain two classes of EGF-binding sites: a high-affinity site with an apparent dissociation constant (K_D) of 0.7 nM (7.5×10^4 sites per cell) and a low-affinity site with a K_D of 8.5 nM (1.9×10^6 sites per cell). Non-equilibrium binding studies revealed the existence of two kinetically distinguishable sites: a fast-dissociating site, with a dissociation rate constant (k_{-1}) of 1.1. $\times 10^{-3}s^{-1}$ ($1.0–1.3 \times 10^6$ sites per cell) and a slow-dissociating site, with a k_{-1} of $3.5 \times 10^{-5}s^{-1}$ ($0.6–0.7 \times 10^6$ sites per cell).

The cytoskeleton of A431 cells was isolated by Triton X-100 extraction. Scatchard analysis revealed that ~5% of the original number of receptors were associated with the cytoskeleton predominantly via high-affinity sites (K_D = 1.5 nM). This class of receptors is further characterized by the presence of a fast-dissociating component ($k_{-1} = 2.0 \times 10^{-3}s^{-1}$) and a slow-dissociating component ($k_{-1} = 9.1 \times 10^{-5}s^{-1}$). The distribution between fast and slow sites of the cytoskeleton was similar to that of intact cells (65% fast and 35% slow sites). Incubation of A431 cells for 2 h at 4°C in the presence of EGF resulted in a dramatic increase in the number of EGF receptors associated to the cytoskeleton. These newly cytoskeleton-associated receptors appeared to represent low-affinity binding sites (K_D = 7 nM). Dissociation kinetics also revealed an increase of fast-dissociating sites. These results indicate that at 4°C EGF induces the binding of low-affinity, fast-dissociating sites to the cytoskeleton of A431 cells.

Key words: Scatchard analysis, dissociation kinetics, epidermal growth factor, binding analysis, Triton X-100 extract

Epidermal growth factor (EGF), a polypeptide growth factor, acts on its target cells first by binding to receptors located on the cell surface followed by the initiation

Received February 1, 1988; accepted November 18, 1988.

of a number of characteristic responses and, depending on the cell system, this binding leads ultimately to initiation of DNA synthesis and cell division [for reviews see 1–4].

Among the effects of EGF on its target cells are morphological changes, such as rounding up of cells [5], induction of membrane ruffling, extension of filopodia [6,7], and redistribution of actin and α-actinin [8]. Since cell morphology is largely maintained by an integrated action of cytoskeletal systems [9], these results suggest that EGF causes changes in the organization of the cytoskeleton via a direct or indirect coupling. In this respect it is of interest that the EGF-receptor kinase has been demonstrated to be associated with the Triton X-100-insoluble cytoskeletons of A431 cells [10]. Furthermore, in these cells the EGF receptor appeared to be structurally associated with the cytoskeleton, as demonstrated by a variety of electronmicroscopic methods [11].

The cytoskeleton-associated fraction of the EGF receptors represented specifically the high-affinity class of receptors, as deduced from Scatchard analysis of EGF binding [11]. These results were supported by the observations that the high-affinity class of EGF receptors of A431 cells exhibited no lateral mobility [12]. The association of growth factor receptors to the cytoskeleton has been demonstrated also in pheochromocytoma (PC12) cells for both the EGF receptor and the nerve growth factor (NGF) receptor [13–15]. In addition, other receptors have been demonstrated to be associated with the cytoskeleton; these include the N-formylated peptide receptor [16,17], the cyclic AMP receptor [18], and the fibronectin receptor [19].

It has been suggested that the cytoskeleton plays a direct intermediate role in signal transduction exerted by growth factors, ultimately leading to DNA synthesis and cell division [20,21]. Actin filaments have been suggested to be involved in endocytosis [22], while the microtubule system has been demonstrated to regulate intracellular transport of the EGF receptor [23] and the transferrin receptor [24]. The cytoskeleton may thus be involved in intracellular targeting of either the ligand or the receptor or both and may in this way influence signal transduction.

In this paper we present a further characterization of the properties of cytoskeleton-associated EGF receptors of A431 cells. It is demonstrated that high-affinity receptors are associated with the cytoskeleton. Addition of EGF to the cells resulted in an additional association of low-affinity, fast-dissociating receptors to the cytoskeleton.

MATERIALS AND METHODS

Cells and Culture Conditions

A431 human epidermoid carcinoma cells were grown in Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal calf serum (Flow Labs., Irvine, Scotland) in a 7% CO_2 humidified atmosphere at 37°C. Cells were grown routinely to a final density of 50,000–100,000 cells/cm^2.

Isolation of Cytoskeletons of A431 Cells

Cytoskeletons of A431 cells were prepared by extraction for 10 min with 0.5% Triton X-100 in 25 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (Hepes) (pH 7.4) and 1 mM phenylmethylsulfonyl fluoride (PMSF) at 4°C. After

removal of the Triton X-100-containing buffer, the cytoskeletons were washed in the same buffer but without Triton X-100.

EGF Binding Studies

Scatchard analysis. Determination of specific binding and Scatchard analysis according to the LIGAND program was performed as described in detail previously [25]. Briefly, A431 cells were grown on tissue culture clusters (16 mm well diameter, Costar Cambridge MA) and washed twice with phosphate-buffered saline (PBS) (pH 7.4). Subsequently, cells or isolated cytoskeletons were incubated in 0.5 ml DME supplemented with 0.1% bovine serum albumin and buffered with 25 mM Hepes (pH 7.4). EGF (receptor grade) was obtained from Collaborative Research Corp. (Waltham, MA) and ^{125}I-EGF from New England Nuclear (Boston, MA). The ^{125}I-EGF was >95% precipitable in trichloroacetic acid. ^{125}I-EGF (100,000 cpm/ng) was added to the cells at varying final concentrations and incubated for 2 h at 4°C, sufficient to obtain steady-state conditions for all EGF concentrations used (data not shown). The cells or cytoskeletons were then washed thrice in PBS and solved in 0.5 ml 1 M NaOH. Bound radioactivity was determined by counting in a gamma counter (Crystal 5412, United Technologies Packard, Downers Grove, IL).

The values for ^{125}I-EGF binding were corrected for non-specific binding by subtracting the amount of radioactivity bound in the presence of 500 ng/ml unlabeled EGF. At equilibrium these values ranged between 1% and 10% of the total binding of intact cells.

EGF dissociation. A431 cells or isolated cytoskeletons were labeled with ^{125}I-EGF (20 ng/ml final concentration) for 2 h at 4°C, as described above. After one wash with PBS, the cells were incubated for various periods of time in 0.25 ml DME containing 200 ng/ml EGF. After two washes with PBS, the radioactivity was determined as described above. The results presented are the mean values of three different experiments; each part of all experiments was performed in triplicate.

Dissociation of a ligand from its receptor can be described according the model of Clark [26] by $B_t = B_o e^{-kt}$. If more receptor classes are involved, the receptor dissociation can be described by $B_t = B_{0,1} \cdot e^{-k-1,1.t} + B_{0,2} \cdot e^{-k-2,2.t} \ldots + B_0 e^{-k-1,n.t}$ [26]. $B_{0,1}, B_{0,2}, \ldots B_{0,n}$ represents equilibrium binding to each of the different components at the onset of dissociation; $k_{-1,1}, k_{-1,2}, \ldots k_{-1,n}$ are the dissociation rate constants of the components 1 to n expressed in s^{-1}. Analysis of the dissociation kinetic data was performed using the EnzFitter program (Elsevier-Biosoft, Cambridge, England) or plotting ln (Bt/Bo) against time; both methods yield similar results.

Protein and Phospholipid Determination

A431 cells were grown in 150 cm^2 culture flasks and suspended in 1 ml of PBS by trypsinization. Various quantities of Triton X-100 were added to 100 μl samples, and the cells were extracted for 10 min at 4°C. After extraction the cells were centrifuged for 2 min at 10,000*g*. Protein and phospholipid determinations were performed both on pellet and supernatant. Protein determination was performed according the BCA–protein assay reagent (Pierce Chemical Company, Rockford, IL). For phospholipid isolation the pellet and supernatant were extracted with chloroform/metha-

nol according to Bligh and Dyer [27], and the phosphate content was determined according to Rouser et al. [28].

RESULTS

Characteristics of Triton X-100 Extraction

The association of EGF receptors with the cytoskeleton has been demonstrated on glutaraldehyde-fixed A431 cells, using 1% Triton X-100 for 30 min [11]. To avoid the artificial effects of fixatives and to allow a study of the dynamics of EGF receptor–cytoskeleton association, we determined EGF binding on cytoskeletons of non-fixed A431 cells.

The effect of various concentrations of Triton X-100 on cellular protein and phospholipid contents in non-fixed A431 cells was studied. As shown in Figure 1, the phospholipid content decreases dramatically after the addition of Triton X-100 and reaches a minimum value after extraction for 10 min at 4°C with 0.5% Triton X-100. Increasing the Triton X-100 concentration only results in a small further decrease of phospholipid content. Protein extraction occurred at much lower detergent concentrations. Addition of 0.05% Triton X-100 for 10 min at 4°C was sufficient to remove 63% of the total protein content (Fig. 1). No further loss of protein was observed at increasing concentrations of Triton X-100. These data demonstrate that the presence of 0.5% Triton X-100 for 10 min at 4°C is sufficient to remove the soluble proteins and vir-

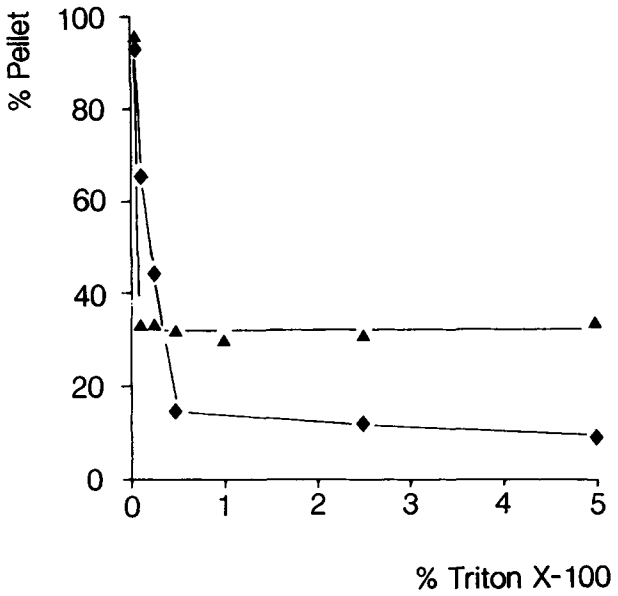

Fig. 1. Effect of Triton X-100 concentration on extraction of proteins and lipids. A431 cells were extracted with different concentrations Triton X-100 for 10 min at 4°C. After extraction, cells were pelleted by centrifugation at 10,000*g* for 2 min and protein and lipid content was determined, both in the supernatant and pellet, as described in Materials and Methods. Percentage protein present in pellet (▲—▲); percentage phospholipid present in pellet (♦—♦).

tually all phospholipids. The residue obtained is defined as the cytoskeletons of A431 cells.

EGF Binding to A431 Cytoskeletons

Binding of EGF to the cytoskeletons of A431 cells is shown in Figure 2. Within a minute of Triton X-100 treatment, a maximal decrease of EGF binding is obtained. No further decrease of EGF binding is observed during prolonged Triton X-100 treatment (Fig. 2).

A similar effect of Triton X-100 was obtained when the cells were incubated with EGF prior to the Triton X-100 extraction. However, in this case the binding of EGF was significantly higher than that measured on isolated cytoskeletons (Fig. 2). These results demonstrate that at least a part of the EGF receptor population is associated with the cytoskeleton of non-fixed A431 cells. Furthermore, the results show that incubation of intact cells with EGF at 4°C results in an increase of Triton X-100-resistant EGF-binding sites, suggesting an EGF-induced association of receptors to the A431 cytoskeleton.

Scatchard Analysis

Receptor characteristics, such as affinity and receptor numbers per cell, are usually obtained by determination of the relationship between specific binding and

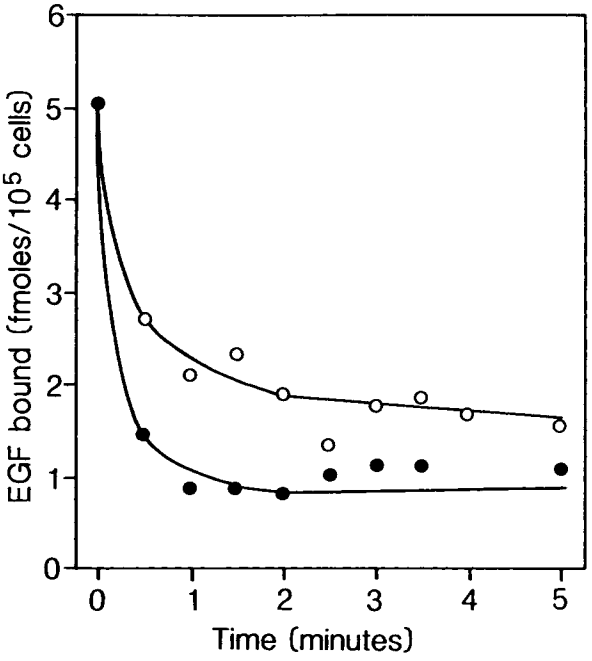

Fig. 2. Effect of Triton X-100 extraction time on EGF binding. The Triton X-100 extraction was performed, as described in Materials and Methods. Specific EGF binding was determined by incubation of intact or Triton X-100-treated cells in the presence of ^{125}I-EGF (2 ng/ml, 100,000 cpm/ng) for 2 h at 4°C, as described previously [25]. Cells treated with Triton X-100 followed by EGF binding (●—●) cells incubated with ^{125}I-EGF, followed by Triton X-100 extraction (○—○).

ligand concentration at steady-state conditions, followed by analysis via the Scatchard method. According to this procedure, A431 cells have been demonstrated to contain two classes of EGF-binding sites; that is, a high-affinity and a low-affinity binding site [11,29], although the precise determination of affinity and numbers of high-affinity sites is considerably hampered by the low number of sites compared with the total number and the relatively small difference between affinities of high- and low-affinity sites [29]. Nevertheless, the best fit of the EGF-binding data plotted following the Scatchard method is according to the two-site model, as shown in Figure 3A.

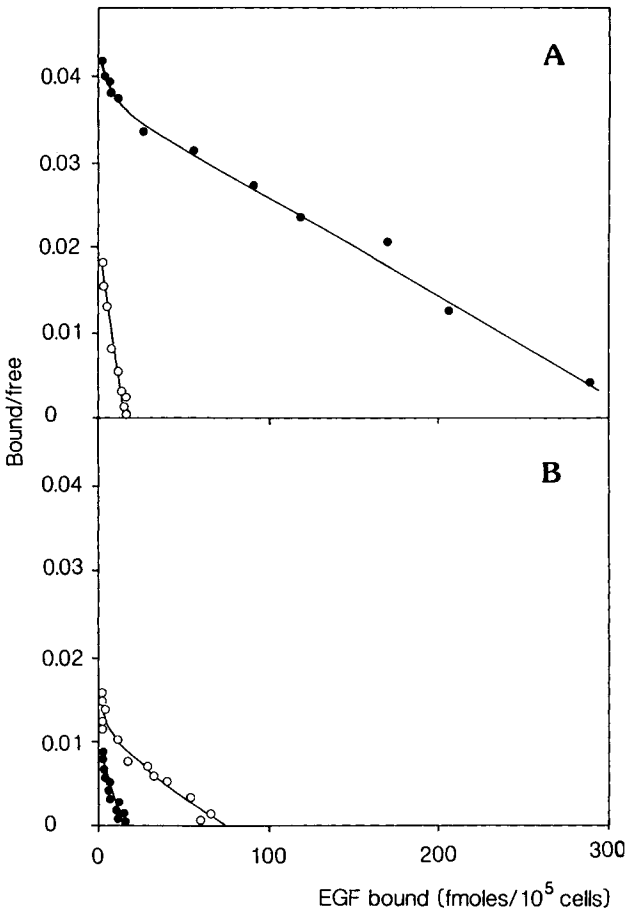

Fig. 3. Scatchard analysis of EGF binding. EGF binding was performed at 4°C for 2 h. The various concentrations of EGF were obtained by mixing ^{125}I-EGF (0.5 ng/ml; 100,000 cpm/ng) with unlabeled EGF, as described in detail previously [25]. Each point represents the mean value of two experiments each performed in triplicate. The data were analyzed using the LIGAND program [25]. **A:** Untreated cells (●—●) and cells preincubated with 40 ng/ml 2E9 (○—○), as described in detail elsewhere [30]. **B:** Cells were extracted with 0.5% Triton X-100 followed by EGF binding, as described in Materials and Methods (●—●), and cells were exposed to EGF as described above, followed by Triton X-100 extraction (○—○).

The high-affinity site is characterized by an apparent dissociation constant (K_D) of ~0.7 nM and 75,000 binding sites per cell, while the low-affinity sites have a K_D of 8.5 nM and 1.9×10^6 binding sites per cell. The reliability of the numbers obtained from Scatchard analysis is demonstrated by the use of the monoclonal anti-EGF-receptor antibody 2E9. This antibody has been demonstrated to specifically block EGF binding to low-affinity binding sites, leaving EGF binding to the high-affinity binding site undisturbed [29]. As shown in Figure 3A, in the presence of saturating amounts of 2E9, EGF binding is characterized by a linear relationship in a Scatchard graph. The K_D is calculated as 0.7 nM, and maximal binding represents 90,000 receptors/cell. These values closely agree with the characteristics of the high-affinity binding site of untreated cells. Isolation of cytoskeletons of non-fixed A431 cells by 0.5% Triton X-100 for 10 min at 4°C, followed by EGF binding also reveals a linear relationship in a Scatchard plot (Fig. 3B), indicating that cytoskeletons exhibit only one class of EGF-binding sites. This class of binding sites is characterized by a K_D of 1.5 nM and 70,000 sites per cell, thus resembling strongly the high-affinity sites of intact cells. As such, these data agree with the presence of high-affinity EGF-binding sites in cytoskeletons of glutaraldehyde-fixed A431 cells [11].

As shown in Figure 2, incubation of intact cells in the presence of EGF, followed by Triton X-100 extraction, results in an increase of Triton X-100-resistant EGF binding, suggesting an EGF-induced association of receptors with the cytoskeleton. Therefore, the EGF-binding characteristics were determined on cytoskeletons of non-fixed cells isolated after the binding period. Analysis of the Scatchard plot clearly reveals the presence of two classes of binding site, that is, a high-affinity site with a K_D of 0.6 nM and 20,000 receptors per cell and a low-affinity site with a K_D of 7 nM and 380,000 receptors per cell (Fig. 3B). These results demonstrate that EGF causes an increase in the association of EGF receptors with the cytoskeleton, primarily due to low-affinity receptors.

Effect of Triton X-100 Extraction on EGF-Dissociation Characteristics

Steady-state binding experiments, as described above, provide important information on receptor characteristics, but information on receptor dynamics, that is, the rate at which the ligand is bound to or dissociated from the receptor is lacking. Thus we have performed a further characterization of cytoskeleton-associated EGF receptors by studying EGF dissociation.

A431 cells were incubated in the presence of 20 ng/ml ^{125}I-EGF for 2 h at 4°C, after which the cells were washed with binding buffer. EGF dissociation was initiated by adding 200 ng/ml unlabeled EGF in binding buffer. The dissociation rate was considerably increased by the addition of excess unlabeled EGF (data not shown). Since adding higher concentrations of EGF (0.5 or 1 μg/ml) did not alter the dissociation rate further, we used an EGF concentration of 200 ng/ml for all dissociation experiments. Plotting $\ln(B_t/B_o)$ against time results in a curvilinear relationship (Fig. 4), indicating that dissociation of EGF is not simply from one single class of receptors but, most likely, from two different classes. Dissociation of EGF from the fast component is characterized by a dissociation rate constant k_{-1} of $1.1 \times 10^{-3} s^{-1}$ and from the slow component by a k_{-1} of $3.5 \times 10^{-5} s^{-1}$. Furthermore, the distribution of EGF is determined as 64% and 36% in the fast and slow sites, respectively. The same distribution

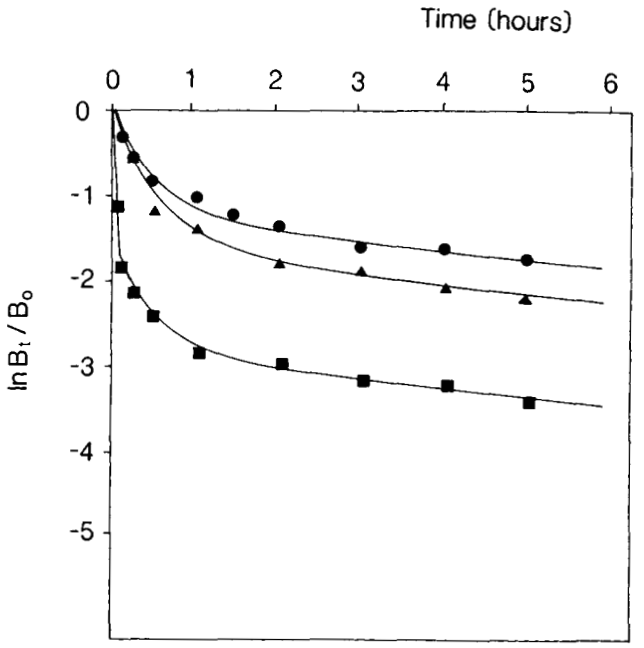

Fig. 4. Dissociation of EGF from its receptor. A431 cells were exposed to 20 ng/ml ^{125}I-EGF (10,000 cpm/ng) for 2 h at 4°C. Subsequently, the dissociation of EGF from its receptor was determined in the presence of 200 ng/ml unlabeled EGF, as described in Materials and Methods. Each point represents the mean value of at least three experiments performed in triplicate. Control cells (●—●); isolated cytoskeletons (▲—▲); dissociation from cytoskeletons, isolated after EGF binding to intact cells (■—■).

between fast and slow receptors is found when labeling is performed under saturating conditions (100 ng/ml EGF) (data not shown).

Subsequently, EGF dissociation has been determined from A431 cytoskeletons. After the Triton extraction, 0.5% Triton X-100 for 10 min at 4°C, the isolated cytoskeletons were incubated in the presence of 20 ng/ml ^{125}I-EGF for 2 h at 4°C, and EGF dissociation was determined, as described in Materials and Methods. As shown in Figure 3B, cytoskeletons contain predominantly high-affinity EGF-binding sites, but, surprisingly, the dissociation curve demonstrates clearly a curvilinear relationship comparable to control cells (Fig. 4). The rate constants of the fast- and slow-dissociation sites are determined as $2.0 \times 10^{-3}s^{-1}$ and $9.1 \times 10^{-5}s^{-1}$, respectively, while the distribution of EGF in the slow and fast compartments is 35% and 65%, respectively. These results demonstrate clearly that dissociation of EGF from cytoskeleton-associated receptors is identical to that of control cells and, furthermore, that the slow- and fast-dissociation sites are not simply identical to the high- and low-affinity receptors, respectively.

Finally EGF dissociation was determined from cytoskeletons of A431 cells isolated after the EGF binding. The EGF receptors associated to these cytoskeletons are characterized by both low- and high-affinity binding sites (Fig. 3B). Analysis of the dissociation data reveal again fast- and slow-dissociation sites; the dissociates rates are

$6.8 \times 10^{-3}s^{-1}$ and $6.1 \times 10^{-5}s^{-1}$, respectively, (Fig. 4). Compared with intact cells and isolated cytoskeletons, a clear difference is observed in the distribution of the two dissociation sites: 90% of the total receptor population associated with the fast site and 10% with the slow site, respectively. Thus isolation of cytoskeletons after EGF binding results in the appearance of low-affinity, fast-dissociating binding sites associated with the cytoskeleton (Figs. 3B and 4).

DISCUSSION

In this paper we have further characterized the properties of EGF receptors associated with the cytoskeletons of A431 cells and present evidence in favor of an EGF-induced association of receptors with the cytoskeleton.

Recently, we demonstrated that high-affinity EGF receptors were associated with the cytoskeleton of glutaraldehyde-fixed A431 cells [11]. Here we also demonstrate that non-fixed A431 cells exhibit high-affinity receptors that were resistant to Triton X-100 treatment. Binding of EGF to intact cells at 4°C followed by Triton X-100 extraction reveals that, under these conditions, additional low-affinity receptors are associated with the cytoskeleton. These observations demonstrate that EGF induces an association of EGF receptors with the cytoskeleton. These observations were obviously not obtained when the cells were pre-fixed with glutaraldehyde [11].

Dissociation of EGF from its receptors reveals the presence of two classes of sites, a fast- and a slow-dissociating site. The fast sites represent 64% and the slow sites 36% of the total receptor population independent of the EGF concentration used for pre-labeling.

It is tempting to suggest that slow-dissociating sites are identical to the high-affinity binding sites and fast-dissociating sites to the low-affinity binding sites. However, the ratio between occupied high- and low-affinity receptors at steady-state conditions depends on the EGF concentration [29]. In contrast, the ratio between occupied slow and fast receptors in intact cells appears to be independent of the EGF concentration (unpublished observations), demonstrating that the high-affinity sites obtained in a Scatchard graph are not simply identical to the slow-dissociating sites obtained in a dissociation assay.

Based on the numbers obtained in this study (~5% high-affinity receptors and 35% slow-dissociating sites) it can be concluded that the quantity of slow sites is strongly over-estimated, most likely due to a re-association of released ligand with the receptor during the dissociation assay.

In this respect, the dissociation of EGF from isolated cytoskeletons also should be considered with caution, since cytoskeletons exhibit apparently only one class of high-affinity binding sites (Fig. 3B) but clearly two classes of dissociating receptors. These conflicting results may be due to either the presence of a small fraction of low-affinity binding sites associated with the cytoskeleton (not detectable in a Scatchard graph), a re-association of released EGF to the receptors during the dissociation assay, or both. The presence of a small fraction of low-affinity sites on isolated cytoskeletons is supported by the observation that the K_D is calculated as 1.5 nM, a twofold increase compared with the K_D of the high-affinity binding site. These findings clearly demonstrate that a quantitative comparison of binding characteristics obtained from equilib-

rium and non-equilibrium studies requires a further detailed study of the mechanisms of association and dissociation under experimental conditions.

Finally, binding of EGF at 4°C to intact cells followed by Triton X-100 extraction and EGF dissociation demonstrates a seven- to eightfold increase in the number of fast-dissociating sites. Although a quantitative comparison between the data obtained in Figures 3 and 4 appears not to be justified, a qualitative comparison demonstrates that EGF causes association of a relatively high number of low-affinity binding sites with the cytoskeleton and a significant increase in the relative number of fast-dissociating sites. Therefore it is concluded that the novel cytoskeleton-associated EGF receptors are both low-affinity and fast-dissociating ones.

An important aspect of the interaction between the EGF receptor and the cytoskeleton involves the nature of the cytoskeletal component. Previously, it has been suggested that actin is involved in this association, as judged by the diameter of the elements involved in electron micrographs [11]. Recently, this has been supported by co-localization of EGF receptors and actin filaments by immunofluorescence microscopy (manuscript in preparation). It is tempting to suggest that this interaction between EGF receptors and the actin filaments is involved in receptor clustering, as demonstrated quantitatively in A431 cells and, consequently, specifically involved in receptor mediated endocytosis. For intracellular transport, the microtubule system—not actin—seems to be more important [23,24]. It is clear that further analysis of the characteristics of the EGF receptor, including cytoskeletal association, level of phosphorylation, and clustering, is required for a full understanding of the molecular mechanism underlying the activation of signal transduction by EGF.

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