Epidermal Growth Factor Receptors Associated to Cytoskeletal Elements of Epidermoid Carcinoma (A431) Cells

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Abstract. The structural interaction of the epidermal growth factor (EGF) receptor and the cytoskeleton of A431 cells has been studied using a monoclonal anti-EGF receptor antibody. This has been done with immunogold labeling using a variety of electron microscopical preparation procedures and EGF binding studies. By providing an image of the membrane-associated cytoskeleton, the dry cleavage method reveals a preferential localization of EGF receptors superimposed upon cytoskeletal filaments. The co-localization of gold particles with cytoskeletal filaments is not affected when pre-labeled cells are extracted with the non-ionic detergent Triton X-100, as visualized by dry cleavage. Using surface replication, this treatment results in visualization of the cytoskeleton. In these latter preparations, it is also observed that EGF receptor-coupled gold particles remain associated with cytoskeletal elements. Moreover, Triton extraction performed before immunogold labeling of EGF receptors demonstrates that isolated cytoskeletons contained binding sites for anti-EGF receptor antibodies. Using stereo micrographs of replica's obtained from these isolated cytoskeletons, it is shown that gold-labeled EGF receptors are exclusively present on the cortical membrane-associated region of the cytoskeleton and not on more intracellular-located filaments.

Scatchard analysis of EGF binding to cells fixed with glutaraldehyde and treated with Triton X-100 before and after EGF binding indicates that a high affinity EGF binding site is associated with the Triton X-100 insoluble cytoskeleton.

EPIDERMAL growth factor (EGF)[1] is one of the most intensively studied polypeptide growth factors, and detailed knowledge has been obtained of the various effects of EGF in its target cells as well as of the molecular characteristics of EGF and EGF receptor (for reviews see references 7, 10, 27). Among the effects of EGF in its target cells are morphological changes, such as rounding up of cells (9) and induction of membrane ruffling and extension of filopodia (8). Furthermore, it has been shown that EGF causes alterations in the distribution of actin and α-actinin (26). Since morphology and dynamics of the cell are largely maintained by an integrated action of cytoskeletal systems (2, 12), these observations suggest that EGF causes changes in the organization of the cytoskeleton via a direct or indirect coupling. Interestingly, an association of the EGF receptor kinase with the Triton X-100-insoluble cytoskeleton of A431 cells has been described recently (17). Furthermore, an enhanced phosphorylation of high molecular weight cytoskeletal proteins was determined as a consequence of EGF-induced kinase activity (17). In addition to the A431 cells, binding of EGF to purified cytoskeleton of the pheochromocytoma cell line PC12 has also been demonstrated (29).

So far, no morphological evidence at the ultrastructural level has been presented for an interaction of EGF receptors with the cytoskeleton. The recent development of a number of electron microscopical techniques and immunolabeling methods, however, allows for such an ultrastructural study. These techniques include the preparation of rotary-shadowed platina/carbon replicas of normal, as well as detergent-extracted, critical point-dried cells (6, 15) and the dry cleavage technique of Mesland (18, 24).

In this paper, we have studied the interaction of the EGF receptor with the cytoskeleton of A431 cells using the above-mentioned methods. The apparent homogenous distribution of these receptors on the cell surface, which has also been reported previously (4), appears to be a preferential localization of receptors in the immediate vicinity of the membrane-associated filaments. Furthermore, it is shown by performing Triton extraction, either before or after incubation with anti-EGF receptor antibody, that at least a part of the EGF receptor population is indeed structurally associated with the cytoskeleton. These data were confirmed by Scatchard analysis of EGF binding on both normal and detergent-extracted cells as well as by the correlation between the number of gold particles per μm^2 cell surface and the label efficiency as measured with radioactively labeled EGF.

1. *Abbreviation used in this paper*: EGF, epidermal growth factor.

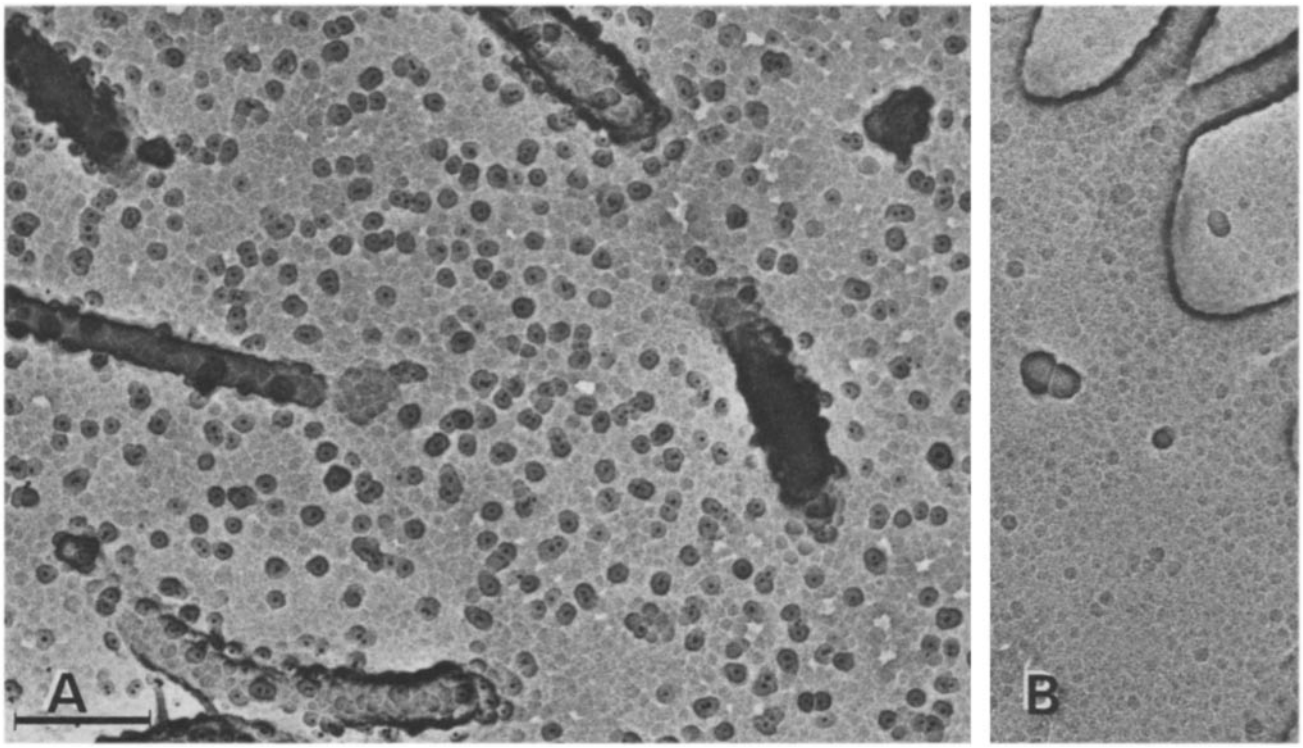

Figure 1. (*A*) Cell surface distribution of EGF receptors on A431 cells as visualized by surface replication. The cells were incubated with the anti-EGF receptor antibody (2E9), which was visualized using a second antibody and protein A-coated gold particles, with a diameter of 10 nm. Bar, 0.25 μm. (*B*) Cell surface of non-treated control cells.

Materials and Methods

Cell Culture

A431 human epidermoid carcinoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Flow Labs, Irvine, Scotland) in a 7% CO_2 humidified atmosphere at 37°C.

Cells were grown to ±70% confluency in a serum-containing medium for 24 h, followed by a 16-h period in serum-free medium.

Immunolabeling

A431 cells were fixed with 2% formaldehyde and 0.1% glutaraldehyde in 60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM $MgCl_2$, pH 6.9 (31), for 15 min. After brief rinsing, the fixative was quenched with 50 mM glycin and/or 0.5 mg/ml sodiumborohydride. Subsequently, the cells were consecutively labeled with anti-EGF receptor antibody, designated 2E9, rabbit anti-mouse antibody, and protein A-gold conjugates, essentially as described previously (4). Then the cells were extensively rinsed, followed by postfixation with 0.25% glutaraldehyde for 15 min. Detergent extraction of formaldehyde-fixed cells (30 min with 1% Triton X-100) was performed either before or after incubation with anti-EGF receptor antibodies as indicated in the Results section.

Table I. Effect of Triton Extraction on Gold Particle Density

	Number of gold particles/μm²		
	Control	A	B
Replicas	125 ± 25	116 ± 19	112 ± 25
Dry Cleavage	114 ± 27	106 ± 24	Not done

Dry cleavage and surface replication were used to measure the gold particle density which is given as the number of gold particles/μm² ± SD. The control values can be compared with (*A*) the effect of Triton X-100 on EGF receptor-labeled cells, and (*B*) the gold particle density, obtained when cells were first detergent-extracted and subsequently labeled with anti-EGF receptor antibodies (2E9). Data were corrected for nonspecific binding.

(The number of nonspecific gold particles on the surface replica of both control and Triton-extracted cells per μm² surface area was found to be 3 and 14, respectively.) The number of gold particles on at least 30 μm² of surface area was counted (~2 μm² per cell) and expressed as the number of gold particles per μm². The cells were randomly chosen from three separate experiments.

Surface Replication

A431 cells were grown to near confluency on glass coverslips. The cells were immunolabeled as described above, followed by postfixation for 15 min using 0.5% OsO_4. Dehydration, critical point drying, and preparation of rotary-shadowed platina/carbon replicas was performed according to standard procedures. Replicas were floated off on hydrogen fluoride and washed in distilled water. Organic material was removed on 15% sodiumhypochlorite for 1 h, washed three times in distilled water, and picked up on grids. Replicas of the cytoskeleton were obtained by rotary shadowing detergent-extracted cells (6, 15).

Dry Cleavage of Cells

Cells were grown to near confluency on tissue culture dishes (35-mm diameter, Costar, Cambridge MA). To study the medium-directed side of the cell, part of the glutaraldehyde-fixed confluent cell monolayer was detached and inverted as described (24). The inverted cell layer was attached to a poly-L-lysine-coated (2 mg/ml, M_r 150,000-300,000), formvar-carbon-covered 300 mesh Nickel grid (VECO, The Netherlands) (20). After postfixation with 0.5% OsO_4 during 15 min, cells were dehydrated and critical point dried, according to standard procedures. Dried cells on grid were cleaved by means of adhesive tape (18). The grid was positioned, cell-side down on the adhesive side of a clean piece of Scotch tape, and gently pressed. Subsequently, the grid was removed with forceps (while watching it with a binocular microscope), cleaving the cells and leaving the largest part of each cell on the tape (18). The medium-directed cell membrane with its associated cytoskeleton remained on the grid and was immediately examined in the electron microscope or stored in a dry environment.

EGF Binding Studies

Cells were grown on tissue culture clusters (16-mm well diameter, Costar, Cambridge MA) to near confluency, washed twice with phosphate-buffered saline (pH 7.4). Subsequently, cells were incubated in 0.5 ml DME supplemented with 0.1% bovine serum albumin and buffered with 25 mM

Hepes (pH 7.4). ^{125}I-EGF (100,000 cpm/ng) or ^{125}I-2E9 (200,000 cpm/ng) was added to the cells followed by an incubation for 2 h at room temperature. Then the cells were treated as described above in Surface Replication, and radioactivity was determined using a gamma counter (Packard Autogamma 5650), as described previously (5). The values for ^{125}I-binding were corrected for nonspecific binding by subtracting the amount of radioactivity bound in the presence of excess unlabeled ligand.

Scatchard analysis was performed using a modified version of the LIGAND program, as was described in detail previously (5).

Materials

The monoclonal anti-EGF receptor antibody, designated 2E9, was raised and characterized as described in detail elsewhere (Defize, L. H. K., W. H. Moolenaar, P. T. van der Saag, J. Boonstra, and S. W. de Laat, manuscript submitted for publication). 2E9 was shown to be directed against an amino acid sequence located at or close to the EGF binding domain. Rabbit anti-mouse antibody (7S) was obtained from Nordic (Tilburg, The Netherlands). Protein A–coated gold particles with a diameter of 10 nm were used. ^{125}I-EGF was obtained from New England Nuclear (Boston, MA) and ^{125}I-2E9 was prepared by the Chloramine T method.

Results

Cell Surface–located EGF Receptors Visualized by Surface Replication and Dry Cleavage

A typical surface labeling as shown in Fig. 1 *A* was obtained upon incubation of A431 cells with monoclonal anti-EGF receptor antibodies (2E9), followed by treatment of cells for immunogold labeling and surface replication as described in Materials and Methods. The cell surface is densely covered with gold particles, demonstrating an apparent homogenous distribution of EGF receptors, which has been reported previously with the freeze etch and the label fracture method as well (4). In experiments in which the 2E9 antibody was omitted during the labeling procedure, virtually no gold particles were observed on the replicas (Fig. 1 *B*, Table I) demonstrating the specificity of the labeling.

The dry cleavage method has been originally developed to visualize the plasma membrane–associated cytoskeleton of the substrate-adherent side of the cell (18). Recently, this method has been modified to allow a visualization of the submembraneous cytoskeleton of the medium-directed side of cells (24). This modified dry cleavage method in combination with immunogold labeling has been used to visualize EGF receptors superimposed on the submembraneous cytoskeleton of A431 cells. The cytoskeletal structures visualized by this method include most likely stress fibers and microfilaments (18–20) (Fig. 2). The apparent homogenous distribution of immunogold-labeled receptors (Fig. 1) results from a preferential localization of gold particles in the immediate vicinity of intracellular-located filaments, near or attached to the plasma membrane. Virtually no gold label has

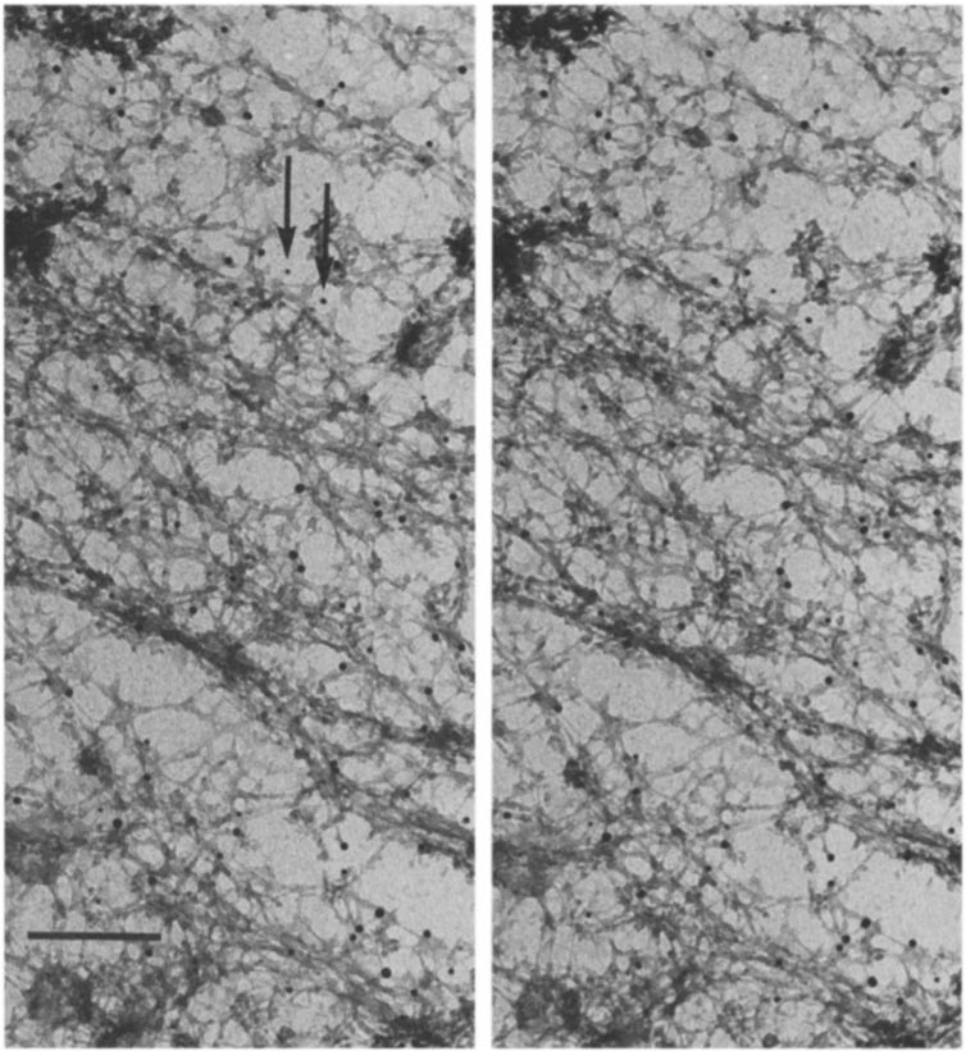

Figure 2. A stereo pair of the distribution of gold-labeled EGF receptors in relation to the submembraneous located cytoskeleton as visualized with dry cleavage. EGF receptors were labeled with the monoclonal antibody 2E9. Cells were subsequently fixed and inverted on poly-L-lysine–coated grids, in order to visualize the medium facing cell side (see Materials and Methods). The stereo micrographs were taken at tilt angles of 6°. The viewing direction is from inside the cell through the membrane-associated filamentous network towards the membrane of the medium-facing surface. In these preparations, the plasma membrane is not visible, since the membrane is not electron dense enough. The presence of the membrane in these preparations has been demonstrated by thin sectioning as described previously (18, 24). Note that the 10-nm gold-labeled EGF receptors colocalize with intracellular filaments except for the two indicated gold particles (arrows). Bar, 0.25 μm.

been found in membrane areas overlying openings in the cytoskeletal network (except for two gold particles, which are indicated in Fig. 2). These results suggest a structural coupling between the EGF receptors and cytoskeletal elements in cultured A431 cells.

Effect of Triton Extraction

To establish whether the observed topographical relation between EGF receptor and membrane-associated cytoskeletal elements is coincidental or based upon a structural connection, the effects of Triton X-100 extraction were studied. Cells were fixed, labeled with 2E9, and subsequently treated with Triton X-100. After completing the immunogold labeling procedure, replicas of the remaining cytoskeleton were made (Fig. 3). The effect of Triton X-100 is clearly demonstrated by comparing Figs. 1 and 3. Numerous gold particles can be detected on these Triton-extracted cells. Stereo micrographs demonstrate that the gold label is localized exclusively in the plasma membrane–associated region (Fig. 3). Omission of the 2E9 antibody from the labeling sequence on extracted cells demonstrated only a small nonspecific labeling of rabbit anti-mouse antibody and protein A–coated gold particles inside the cell (Table I). A structural association of the EGF receptor to the cytoskeleton is also apparent on Triton-extracted dry cleavage preparations (Fig. 4). The remaining filamentous structures are very similar to those obtained from cells not treated with the detergent (compare Figs. 2 and 4).

2E9 Binding on Purified Cytoskeletons

The results described above strongly suggest a structural relationship between EGF receptors and cytoskeletal elements in A431 cells. However, it could be argued that the glutaraldehyde fixation performed before 2E9 binding causes a chemical cross-linking between receptors and cytoskeleton. Therefore, cells were fixed mildly using the non–cross-linking fixative formaldehyde only, followed by Triton extraction, as described under Materials and Methods. After Triton extraction, the remaining cytoskeletons were allowed to bind 2E9, followed by immunolabeling as described. As shown in Fig. 5, replicas of the purified cytoskeletons indeed contained gold particles, indicating the presence of EGF receptors. Moreover, the gold particles were present exclusively on the cell surface–located cytoskeletal filaments, whereas the filamentous structures located more inside the cell are virtually devoid of gold particles (Fig. 5).

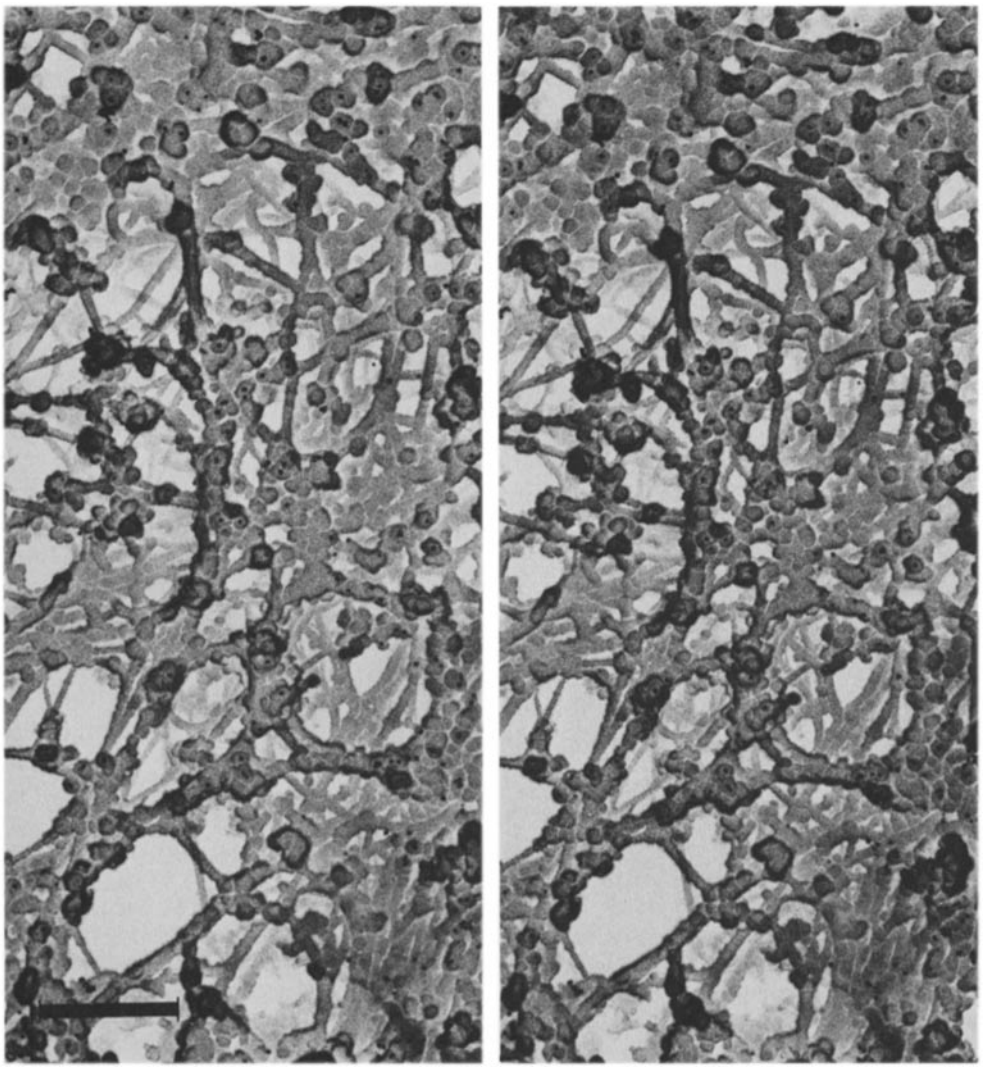

Figure 3. A stereo pair of the cytoskeleton of an EGF receptor–labeled and Triton-extracted cell, as visualized by the surface replication technique. Triton extraction reveals the cytoskeletal filaments. Note the absence of gold label on more intracellular located cytoskeletal filaments. Cells were fixed and incubated with the anti-EGF receptor antibody, and subsequently detergent extracted. After further treatment (see Materials and Methods) cells were dehydrated, critical point dried, and surface replicated. Bar, 0.25 μm.

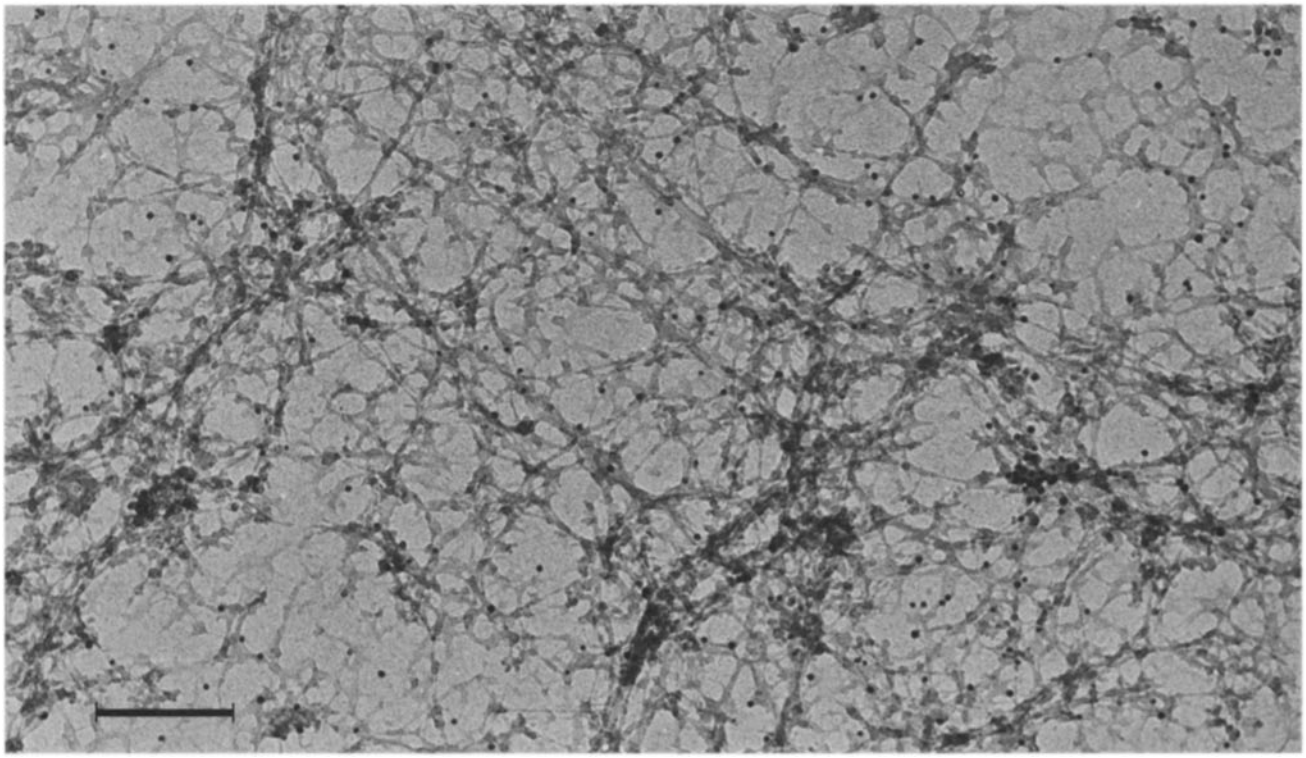

Figure 4. Distribution of gold-labeled EGF receptors on the medium-facing cell side of Triton-extracted cells, as visualized by dry cleavage. In the absence of plasma membrane, the co-localization of gold particles and cytoskeletal filaments is maintained. Immunolabeling (as described in Fig. 2 and in Materials and Methods) was followed by inversion of the labeled cell layer on poly-L-lysine–coated grids, dehydration, critical point drying, and dry cleavage. Bar, 0.25 μm.

Label Efficiency and EGF Binding

An important aspect in all immunoelectron microscopic studies concerns the "label efficiency." In the course of preparation of cell material for examination in the electron microscope, a number of fixation, dehydration, and embedding steps have to be performed after the labeling of a particular protein, and each step might cause loss of label. Therefore, the label efficiency was determined by estimation of gold particle density. A431 cells have been shown to contain approximately two million EGF receptors per cell (11), and assuming a total cell surface area of ~2,800 μm^2 (13), an EGF receptor density of ~715 receptors/μm^2 might be expected. As shown in Table I, all treatments as described above and as shown in Figs. 1–5 yield a gold particle density of ~115 particles/μm^2, being 16% of the total number of EGF receptors. Also, Triton extraction performed either before or after 2E9 antibody incubation does not reduce the number of gold particles per unit of cell surface area. These results indicate that the EGF receptors visualized by the surface replication method (Fig. 1) are all linked to the cytoskeleton.

To establish whether this low number of gold particles is due to inefficient labeling or to a loss of label during the processing of cells for electron microscopic examination, the binding of ^{125}I-EGF and ^{125}I-labeled 2E9 antibody was determined after various treatments. The binding of labeled EGF and 2E9 to untreated viable cells was taken as 100% (Fig. 6 *A*). Pre-fixation with glutaraldehyde caused a severe reduction of binding, both of EGF and 2E9, as has been described previously (3, 4). Glutaraldehyde fixation followed by OsO_4 treatment and dehydration steps, however, results in the loss of almost 90% of EGF or 2E9-binding (Fig. 6 *A*). To establish the effect of Triton extraction on EGF binding, formaldehyde-fixed cells were ^{125}I-EGF labeled, followed by Triton extraction. The pre-fixation with formaldehyde did not influence the amount of bound EGF (Fig. 6 *B*). When formaldehyde-fixed cells were ^{125}I-EGF labeled and subsequently extracted with Triton X-100, only 17% of the initial radioactive value resulted to be Triton insoluble (Fig. 6 *B*). Approximately the same radioactivity was measured, when cells were pre-fixed, Triton extracted, and then incubated with ^{125}I-EGF. These data are in agreement with the gold particle densities as shown in Table I, suggesting that treatment of cells with glutaraldehyde with or without Triton X-100 extraction results in loss of 80–90% of the EGF binding sites.

Furthermore, the EGF binding characteristics of cells treated with glutaraldehyde with or without Triton X-100 extraction has been determined by Scatchard analysis. As shown in Fig. 7 *A*, analysis of the binding data of untreated cells results in a best fit according to the two-affinity site model; i.e., a high affinity site with an apparent dissociation constant (K_D) of 0.83 nM and 4×10^4 binding sites per cell and a low affinity site with K_D of 7.0 nM and 1.6×10^6 sites per cell.

A pre-fixation of the cells with 0.5% glutaraldehyde caused a drastic reduction of the binding (Fig. 6 *A*) and Scatchard analysis (Fig. 7 *B*) revealed a linear relationship, suggesting only one EGF binding site with a K_D of 0.86 nM and 4.7×10^4 sites per cell. Furthermore, treatment of glutaraldehyde-fixed cells with Triton X-100, either after

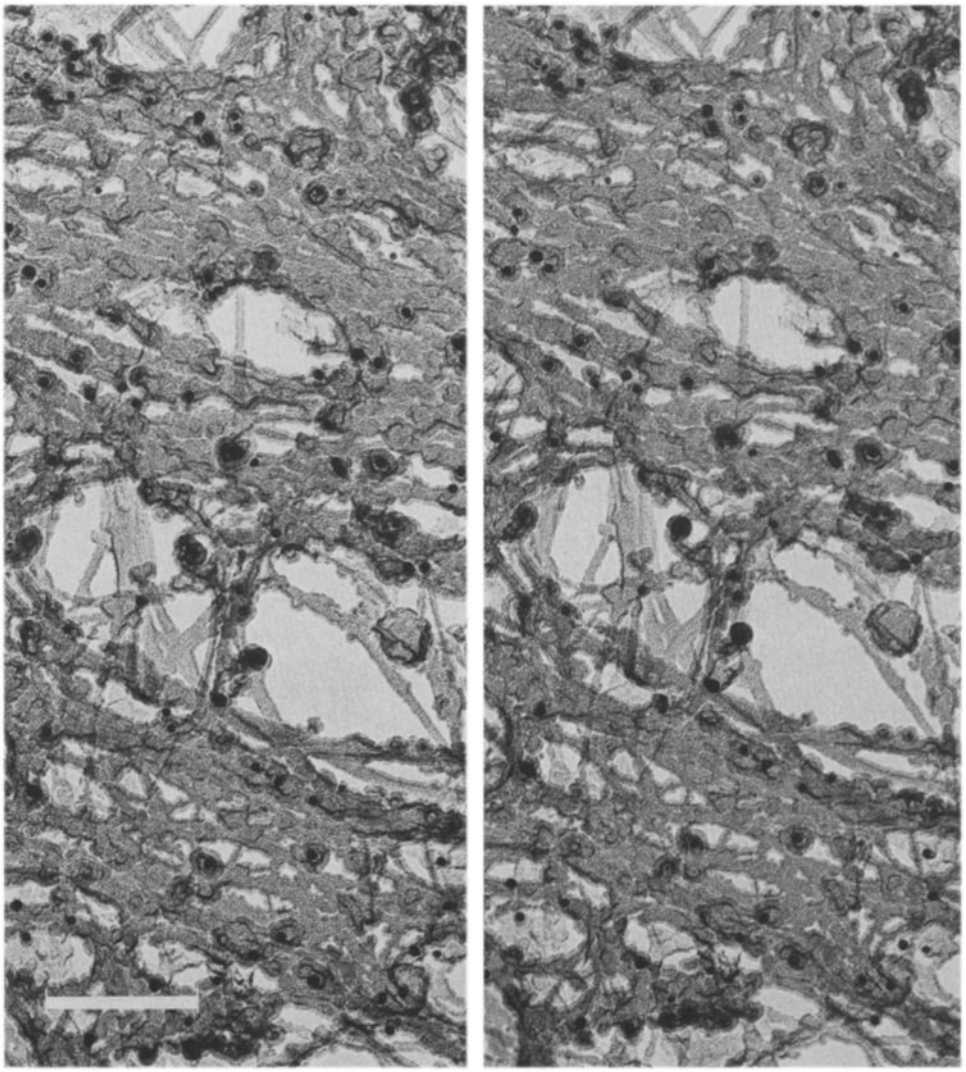

Figure 5. Binding of anti-EGF receptor antibodies (2E9) on Triton-extracted cytoskeletons. After extraction of fixed cells, the cytoskeleton-associated EGF receptors were labeled with 2E9 antibody and visualized by means of surface replication. Note that the gold label is virtually absent on more intracellular-located filamentous structures, as can be seen in stereo pairs. Bar, 0.2 μm.

(Fig. 7 *C*) or before (Fig. 7 *D*) EGF binding resulted essentially in the similar EGF binding characteristics as found in unextracted glutaraldehyde-fixed cells. These results strongly suggest that glutaraldehyde fixation does not affect the high affinity binding sites, but completely inhibits EGF binding to the low affinity binding sites. Furthermore, Triton X-100 extraction does not influence the binding characteristics of glutaraldehyde-fixed cells which again suggests that the high affinity binding sites are associated with cytoskeletal elements.

Discussion

In this paper, ultrastructural evidence has been presented in favor of an interaction between the EGF receptor and cytoskeletal elements in A431 cells. Using the surface replication (15, 23) and the dry cleavage technique (18, 24), it was shown that the apparent homogenous distribution of gold-labeled EGF receptors turned out to be a preferential co-localization of EGF receptors and cytoskeletal filaments. As such, these data are in agreement with the observed co-localization of cell surface glycoproteins with the membrane-associated filament network as was recently reported by Roos et al. (24). Furthermore, by performing Triton extraction on EGF receptor–labeled cells, it was demonstrated that the observed co-localization was indeed based upon a structural interaction.

An interesting feature appears on the surface microstructure of surface replicas (Fig. 1). The gold particles appeared to be almost exclusively located on small bumps. Comparable structures have been previously described to represent membrane proteins (1). In our preparation, the bumps were significantly less pronounced in the absence of the immunolabeling (Fig. 1 *B*). Furthermore, a complex of anti-EGF receptor antibody, rabbit anti-mouse antibody, and protein A–gold attached on poly-L-lysine-coated glass coverslips was shown to cause similar bumps. Therefore, it is suggested that the bumps in our preparations originate mainly from the external labeling of the cells.

Since glutaraldehyde has been used as a fixative before labeling of the EGF receptors, the apparent linkage between EGF receptor and cytoskeleton might be due to the cross-linking activity of glutaraldehyde. Therefore, glutaraldehyde was omitted from the fixation solution and only formaldehyde was used as a fixative. Formaldehyde is known to be unable to induce cross-linking between proteins within the ex-

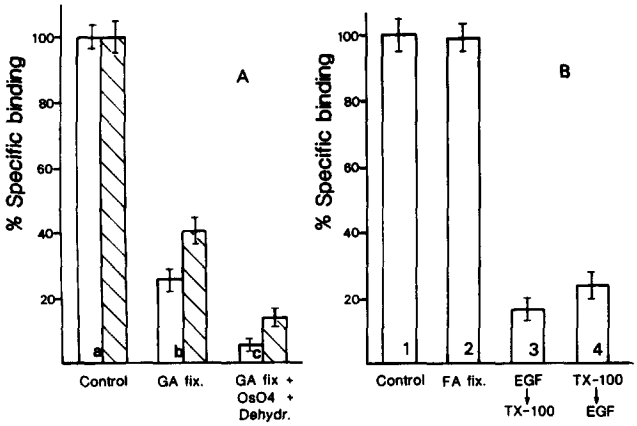

Figure 6. (*A*) Effect of fixation on binding efficiency of ^{125}-EGF (□) and ^{125}I-2E9 (▨). Cells were either glutaraldehyde (*GA*)-fixed (0.1%) (bar *b*) or glutaraldehyde-fixed followed by OsO_4 fixation (0.5% for 15 min) and dehydration (bar *c*). Unfixed control values were taken as 100% (bar *a*). (*B*) Effect of detergent extraction on binding of ^{125}I-EGF to A431 cells. Detergent extraction was performed either before (bar *4*) or after (bar *3*) incubation ^{125}I-EGF on 2% formaldehyde (*FA*)-fixed cells (10 min). FA-fixation did not influence the binding of EGF (bar *2*) as compared with unfixed control cells (bar *1*). Data are presented ±SD.

posure time of 10 min, in contrast to glutaraldehyde (21). After this short pre-fixation, the cells were treated with the detergent Triton X-100 before labeling of the EGF receptors. As shown in Fig. 5, these formaldehyde-fixed, isolated cytoskeletons contained EGF receptors too, located exclusively at the cell surface region. These observations exclude, therefore, artefactual cross-linking between EGF receptors and cytoskeletal elements. In a similar way, a fixation-induced artefactual cross-linkage of concanavalin A binding sites to the cytoskeleton has been excluded (24). Moreover, Landreth et al. (17) showed, without prior fixation, a cytoskeletal association of the EGF receptor in A431 cells using biochemical methods.

An important issue in the application of immunogold labeling methods concerns label efficiency. In this study, the gold particle density has been shown to be similar in both surface replicas and in dry cleavage preparations irrespective of Triton extraction, indicating that all EGF receptors visualized in this study were cytoskeleton associated. Based upon the gold particle density measurements, the labeling efficiency in our study was estimated to be ~16%. It is demonstrated that glutaraldehyde caused a drastic inhibition of EGF binding, probably exclusively due to inhibition of the binding of EGF to the low affinity binding site. Furthermore, EGF binding to formaldehyde-pre-fixed cells (100% label efficiency) followed by Triton extraction also caused a reduction of EGF binding to ~20% of the initial value.

With respect to the nature of these cytoskeleton-associated EGF receptors, the Scatchard plots indicate that the high affinity class of EGF receptors were associated with the cytoskeleton. These data are in agreement with those of Rees et al. (22), who showed that the high and the low affinity class of EGF binding sites on A431 cells had different lateral mobilities. Using fluorescence photobleaching recovery, it was shown that the high affinity EGF receptors (12% of total binding sites) were immobile. This fraction of high affinity EGF receptors might therefore represent the cytoskeleton-associated EGF receptors which we have visualized in this study. In addition, a different cytoskeleton association of the two classes of EGF receptors (29) and of nerve growth factor receptors (25, 30) on PC-12 cells has been reported.

The nature of the filaments to which the receptors are as-

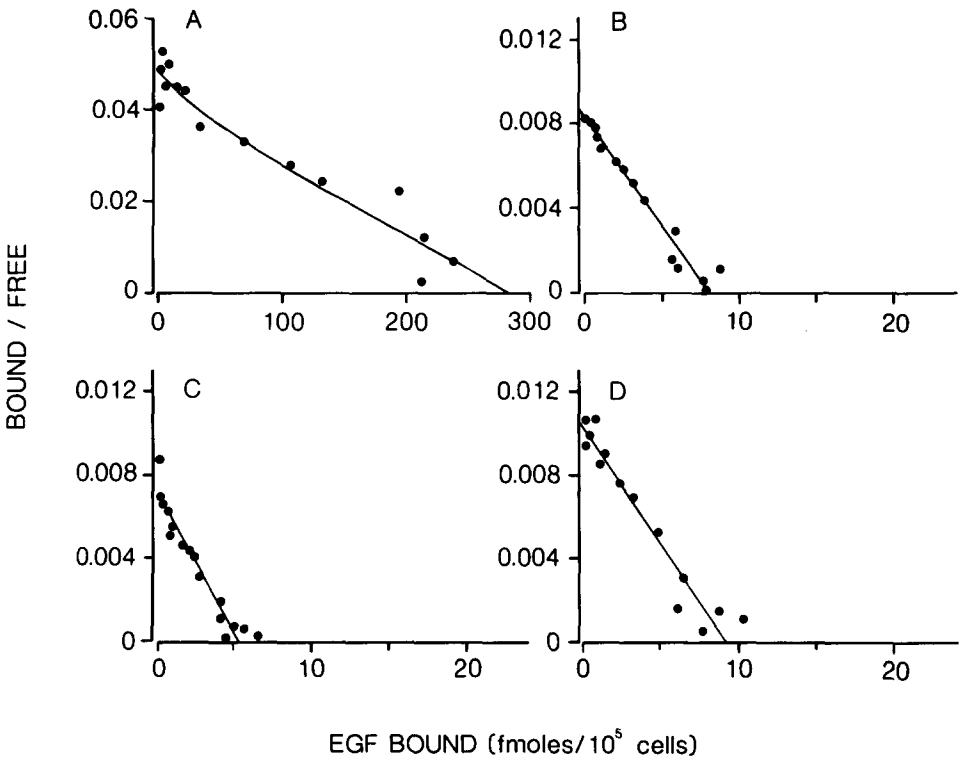

Figure 7. Scatchard analysis of EGF binding in A431 cells. EGF binding has been performed at 4°C for 2 h. The various concentrations of EGF were obtained by mixing ^{125}I-EGF (1 ng/ml; 100,000 cpm/ng) with unlabeled EGF as described in detail previously. Each point represents the mean value of two experiments, each performed in triplicate. The data were analyzed using the LIGAND program (5). (*A*) Untreated cells. (*B*) Cells were fixed using glutaraldehyde during 15 min at room temperature and quenched subsequently (see Materials and Methods), followed by the binding assay as described above. (*C*) Cells were treated as under *B*, after EGF binding the cells were treated with 0.5% Triton X-100 in PBS. (*D*) Cells were fixed as under *B*, followed by Triton X-100 extraction and subsequently EGF binding.

sociated has yet to be determined. Based on a diameter of 6–8 nm in dry cleave preparations, the most likely candidates seem to be actin filaments. A cytochalasin B sensitivity of these membrane-associated filaments has been demonstrated previously (19). Furthermore, an ultrastructural demonstration of interactions between actin filaments and membranes has recently been obtained (14). The observation of a constitutive association of the EGF receptor with structural elements of the cell suggests that these structural interactions might play a role in the lateral mobility modulation and substrate specificity or affinity of the EGF receptor. Association of EGF with its receptor causes activation of various kinases, resulting in a phosphorylation of vinculin (32), high molecular weight cytoskeletal proteins (17), cytoskeleton-associated proteins (16), as well as a phosphorylation of the EGF receptor itself. The stable changes in phosphorylation state that persist in the detergent-insoluble cytoskeleton after binding of EGF (17) might well be involved in association of receptors with cytoskeletal elements and in signal transduction. In this respect, it is of interest that cytoskeletal systems have been suggested to play a role in signal transduction exerted by growth factors, resulting in the ultimate initiation of DNA synthesis (28).

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