PARTICIPATION OF MEMBRANE SKELETON PROTEINS IN AGGREGATION OF EPIDERMAL GROWTH FACTOR RECEPTORS IN A431 CELLS*

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Polyclonal antibody against alpha-spectrin of chicken erythrocytes was prepared. This antibody as well as anti-vinculin and anti-annexin I and II, were used for localization of the antigens in A431 cells during translocation of epidermal growth factor receptors (EGF-Rs) on cell surface. During aggregation of EGF-Rs only spectrin and actin aggregates colocalized with the “capped” receptors in adherent as well as in suspended cells. Physiological implication of spectrin involvement in EGF-Rs redistribution in A431 cells is discussed.

Many cytochemical data demonstrate that cell-surface receptors cross-linked by polyvalent ligands are aggregated on one side of the cell as a “cap” [1, for review see 2]. Cortical cytoplasm in the region of cap formation is enriched in several cytoskeletal proteins such as actin, myosin, fodrin (protein of spectrin family), and alpha-actinin [2-6]. Coaccumulation of cell-surface receptors and submembrane cytoskeletal proteins suggests that the receptors exposed to polyvalent ligands are anchored in cytoskeleton and collected into a cap in result of actin-myosin interactions [2, 3, 7].

In this work we examine the participation of membrane cytoskeletal proteins in capping of EGF-Rs in A431 cells. These cells having the abundant cell-surface EGF-Rs [8], serve as a useful model system for studying the receptor-cytoskeleton interactions [9-12]. Association of EGF-Rs with cytoskeletal filaments, probably with microfilaments, was demonstrated by Wiegant et al. [13]. Recently, we have reported that actin

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1 Abbreviations used: CaM, calmodulin; DAB, 3′3′-diaminobenzidine; DTT, dithiothreitol; EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; GAR-FITC, fluorescein-labeled goat anti-rabbit IgG; kDa, kilodalton; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; SDS, sodium dodecylsulphate; SWAM-FITC, fluorescein-labeled swine anti-mouse IgG.
filaments are cocapped with EGF-Rs in A431 cells [14]. In this respect, actin- 
as well as membrane-binding proteins, i.e. spectrin, vinculin, annexin I and II, 
were assumed to mediate the microfilament-EGF-Rs interactions. Proteins of 
spectrin family named as fodrin, calspertin, non-erythrocyte spectrin, are 
ubiquitous in nonerythroidal cells and similarly to spectrin of mammalian 
erythrocytes link microfilaments with integral proteins of the plasma 
membrane [15, 16]. There are evidences of spectrin-like proteins contribution 
to aggregation of cell-surface receptors in lymphocytes, fibroblasts, and 
Dictyostelium amoeba cells [2, 5, 6, 17, 18]. It has been shown that also 
annexins I and II are able to interact with membranes and F-actin in 
Ca^{2+}-dependent manner [19, 20], whereas vinculin binds microfilament 
bundles to plasma membrane at cell-substratum and cell-cell contacts [21]. 

Using the immunocytochemical technique we demonstrate that in A431 
cells actin and spectrin are the proteins engaged in capping of EGF-Rs.

MATERIAL AND METHODS

Antigens and antibodies. Alpha-spectrin was purified from chicken 
erthrocytes according to Howe et al. and Glenney & Weber [22, 23]. Annexin 
II was isolated from bovine lung by the method of Glenney et al. [20]. The 
isolated proteins were additionally purified by preparative 10% SDS- 
polyacrylamide gel electrophoresis. The bands corresponding to either 
alpha-spectrin or annexin II were excised from the gels and after 
homogenization were emulsified with Freund’s adjuvant and used for 
immunization of rabbits. The alpha-spectrin antibodies were affinity-purified 
according to Talian et al. [24]. Anti-annexin I, anti-vinculin, and monoclonal 
mAb5A9 anti-EGF-R antibodies were kindly provided by Dr. C. Comera, 
Pasteur Institute, Paris, Dr. V. E. Koteliensky, Center of Cardiology, Moscow, 
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Specificity of the antibodies was tested by the immunoblotting technique [25].

Immunocytochemical methods. Human epidermoid A431 cells were grown 
on glass in the mixture of Eagle medium, 10% bovine serum, and glutamine. 
The cells were detached from substratum by treatment with 0.02% EDTA. 
Cell-surface EGF-Rs were localized immunocytochemically using anti- 
-EGF-R and SWAM-FITC antibodies. For localization of intracellular 
proteins by the immunofluorescent technique, the cells were fixed in 3% 
formaldehyde and permeabilized subsequently with 0.1% Triton X-100 [9]. 
Then, the specific antibodies, anti-alpha-spectrin (1:10 dilution), 
anti-vinculin (1:20), anti-annexin I and anti-annexin II (1:100), followed by 
GAR-TRIC, were applied. Actin was detected by staining of the cells with 
rhodamine-labeled phalloidin. All the solutions were prepared on PBS (140 
mM NaCl, 10 mM phosphate buffer, pH 7.4). The samples were examined with 
an OPTON fluorescence microscope. For immunoelectron microscopy the
cells after fixation were dehydrated in series of ethanol and then embedded in EPON 812. Ultrathin sections were treated with ethanolic sodium hydroxide to remove EPON resin [26]; 30 seconds later the sections were labeled with anti-spectrin followed by protein A-gold particles (8 nm in diameter) [27]. The samples were examined with a JEM 100B electron microscope.

RESULTS AND DISCUSSION

Purification of chicken erythrocyte alpha-spectrin

Treatment of chicken erythrocyte ghosts with high ionic strength solution (0.6 M KI) released from membranes high amounts of spectrin. When the extract was loaded onto Sephacryl S-200 column, alpha and beta subunits of spectrin were detected as major polypeptides in void volume fractions (Fig. 1). Subunits of spectrin were separated by affinity chromatography. For this purpose, void volume of Sephacryl column (fractions no. 14-17) were pooled and supplemented with KI and CaCl₂ up to 2 M and 3 mM concentrations, respectively, and applied onto calmodulin-Sepharose column. Alpha-spectrin subunit was eluted from the column with the solution in which 3 mM Ca²⁺

![Gel filtration of the chicken erythrocyte high-salt extract on a Sephacryl S-200 column.](image)

**Fig. 1.** Gel filtration of the chicken erythrocyte high-salt extract on a Sephacryl S-200 column. Left panel — diagram of proteins eluted from Sephacryl S-200 column. The proteins extracted from chicken erythrocyte membranes with the solution of 0.6 M KI, 0.5 mM EDTA, 0.1 mM DTT, and 10 mM Tris, pH 8.0, were loaded onto the column. The column was run at 0.8 ml/min. Right panel — SDS-PAGE of fractions. Lane “s”, protein extract applied to the column. Spectrin (240 and 220 kDa) was eluted in void volume (fractions no. 14-17). Proteins of fraction 27 were 10-fold concentrated before SDS-PAGE analysis.
Fig. 2. Affinity chromatography of alpha-spectrin on a calmodulin-Sepharose column. Spectrin enriched fraction — "s" (Sephacryl S-200 void volume) was applied to CaM-Sepharose column in the presence of 2 M KI and 3 mM CaCl₂. The column was run at 0.5 ml/min. The calmodulin-bound proteins were eluted in the presence of 5 mM EGTA (fractions no. 12-17). SDS-PAGE revealed that pure alpha-spectrin subunit was found in fractions no. 12-14. The next fractions (no. 15-17) contained additionally a polypeptide of 150 kDa, a putative proteolytic fragment of alpha-spectrin.

was replaced by 5 mM EGTA (Fig. 2, fractions no. 12-17). Fractions of the second half of the peak, beside alpha-spectrin, contained additionally a polypeptide of 150 kDa — a well known proteolytic fragment of alpha-spectrin [28].

**Characteristics of antibodies**

Chicken alpha-spectrin is considered the most conservative alpha-spectrin subunit of all spectrin-related proteins [29]. Monospecific antibody directed against this protein was able to recognize alpha-spectrin immunoanalog in many cell types [28, 30]. It reacted specifically with the 240 kDa polypeptide of fresh 0.6 M KI chicken erythrocyte extract (Fig. 3a, b). When the extract was stored for several weeks at −10°C, beside the 240 kDa polypeptide, a new one of 150 kDa appeared, which cross-reacted with anti-alpha-spectrin antibody (not shown). Moreover, the two polypeptides of 240 and 150 kDa were recognized by this antibody in homogenates of A431 cells (Fig. 3d).

The antibodies against human vinculin and annexin I labeled specifically only the 130 and 35 kDa polypeptides, respectively (Fig. 3e, f).

The antiserum against annexin II cross-reacted mainly with the 36 kDa polypeptide and, to a lower extent, with the 68 kDa polypeptide of homogenate.
of A431 cells (Fig. 3g). Thus, the polypeptides of 240, 130, 35, and 36 kDa revealed by immunoblotting analysis, can be considered alpha-spectrin, vinculin, annexin I, and annexin II, respectively.

**Participation of cortical proteins in capping of EGF-Rs in adherent A431 cells**

A431 cells growing on glass surface formed small flat colonies (Fig. 4). In these cells EGF-Rs were distributed uniformly on cell surface (Fig. 4a). Spectrin was located mainly under plasma membrane (Fig. 4b). The similar pattern of cellular distribution was observed for vinculin and annexins (not shown). The membrane-associated EGF-Rs and actin-binding proteins were especially well seen in regions of ruffles and cell-cell contacts (Fig. 4a, b, arrowheads).

Cross-linking of EGF-Rs by anti-EGF-Rs followed by secondary SWAM-FITC antibodies (polyvalent ligands), led to aggregation of the
receptors. After the treatment of EGF-Rs by polyvalent ligands during 30 min at room temperature, the receptors were translocated and formed one large aggregate-cap (Fig. 4c, e, g, i, arrows). Actin and all four actin-binding proteins comigrated with EGF-Rs and were accumulated under plasma membrane in regions where aggregation of EGF-Rs took place. The colocalization of EGF-R caps and aggregates of actin (Fig. 4c, d), spectrin (Fig. 4e, f), vinculin (Fig. 4g, h), annexin I (not shown), and annexin II (Fig. 4i, j) was observed.

**Participation of actin and spectrin in capping of EGF-Rs in suspended cells**

In A431 cells detached from substratum, fractions of actin (Fig. 5b) and spectrin (Fig. 5d) were still located under plasma membrane. These proteins were visualized as a continuous cortical layer that reflected uniform spreading of surface-labeled EGF-Rs (compare Fig. 5b with 5a and Fig. 5d with 5c). On the contrary, vinculin and annexins lost their submembrane localization and were distributed in cytoplasm (Fig. 5j, l). Localization of vinculin and annexins depended therefore on cell attachment to substratum. The dissociation of these proteins from plasma membrane could reflect a reconstruction of cytoskeleton during suspending of the cells [31].

Aggregation of EGF-Rs in suspended cells coincided with accumulation of actin and spectrin in the region of cap formation (compare Fig. 5e with 5f and Fig. 5g, 5h). At the same time, no changes in diffusive distribution of neither vinculin nor annexins were observed (Fig. 5i, j and k, l).

Electron microscopic observations confirmed that in capped cells the spectrin aggregates were located in vicinity to the EGF-Rs accumulated on cell-surface (Fig. 6).

Association of microfilaments with EGF-Rs in A431 cells was visualized by ultrastructural studies [13]. As we demonstrated previously actin filaments take part in capping of EGF-Rs in these cells [14, see also Figs. 4d, 5f in this paper]. The results presented above indicate that among examined actin-binding proteins only spectrin is engaged in capping of EGF-Rs in adherent as well as in suspended cells (Figs. 4 and 5).

It has been demonstrated that spectrin is involved in interactions of actin filaments with cell-surface receptors during such processes as: changes of

Fig. 4. Distribution of EGF-Rs and cytoskeletal proteins in adherent A431 cells. Cell-surface EGF-R was labeled with monoclonal anti-EGF-R followed by SWAM-FITC antibodies (a, c, e, g, i) at 0°C. Cells were fixed either immediately (a, b) or after additional incubation at room temperature (30 min). The fixed cells were permeabilized and stained for actin (d), spectrin (b, f), vinculin (h), and annexin II (j). In noncapped cells colocalization of EGF-R (a) and spectrin (b) is especially well seen in the region of membrane ruffles and cell-cell contacts (arrowheads). Aggregation of EGF-Rs coincides with accumulation of actin (c and d), spectrin (e and f), vinculin (g and h), and annexin II (i and j) in places of caps formation (arrows); a, b, g-j X540; c, d X720; e, f X650
Fig. 6. Immunoelectron microscopy localization of EGF-R and spectrin in suspended A431 cells. EGF-R and spectrin antigens are visualized by gold particles of different diameters (15 nm and 8 nm, respectively). Big arrowhead points to the submembrane aggregate of spectrin which accompanies EGF-Rs accumulated on cell surface (small arrowheads) X55,000

erthrocyte cell shape, exocytosis in chromaffin cells, neurosecretion as well as regulation of exposition of the receptors in neurons [16 and references therein]. The coincident accumulation of EGF-Rs and actin/spectrin aggregates suggests that spectrin may be involved in EGF-Rs-microfilaments interactions during the capping of the receptors in A431 cells.

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Fig. 5. Localization of EGF-R, actin, and actin-binding proteins in suspended A431 cells. Cells were double labeled for EGF-R (left panel) and the proteins (right panel), a-d, Noncapped cells. EGF-Rs regularly distributed on cell surface are visualized as a bright ring (a, c). Actin (b) and spectrin (d) form a continuous layer localized just beneath the plasma membrane. A large pool of these proteins is observed also in the cytoplasm. e-n, Capped cells. During cap formation only actin (f) and spectrin (h) are coaccumulated with EGF-Rs (e, g). Vinculin (j) and annexin-1 (l) are permanently dispersed in the cytoplasm. n-Control cells non-treated with the first antibody X500
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