DEPENDENCE OF ACTIVATION OF THE EPIDERMAL GROWTH FACTOR RECEPTOR ON ITS AFFINITY AND OLIGOMERIZATION

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The tyrosine kinase activity of the epidermal growth factor receptor (EGFR) is essential for transmission of the mitogenic signal, for mitogenesis and for cell transformation [6, 10]. According to the hypothesis of intermolecular activation, developed in Schlesinger's laboratory [3], activation of the tyrosine kinase of the receptors takes place due to allosteric interaction of individual domains of the receptors which have undergone oligomerization. Receptors in an oligomeric state are distinguished by higher affinity, and for that reason binding of the ligand stabilizes EGFR dimers. Meanwhile, a model of intramolecular activation shows that as a result of addition of the ligand to EGFR a special kind of vertical "shift" of the allosteric signal takes place and is transmitted through the transmembrane region to the kinase domain, leading to a conformational change and to activation of the domain [3].

The aim of this investigation was to study correlation between oligomerization of EGFR and changes in their affinity and activation of tyrosine kinase.

EXPERIMENTAL METHOD

Cells of human epidermoid carcinoma of strain A-431 were cultured in medium RPMI (Gibco BRL, USA) with the addition of 10% fetal calf serum ("Vektor" Research and Production Combine) on 24-well plastic plates. The cells were fixed with 10% formalin in phosphate buffer (pH 7.4) for 5 min at room temperature.

EGF, isolated from the submandibular salivary glands of mice [1] were labeled with ^{125}I, using chloramine tea, by Greenwood's method [5] (specific activity 4 MBq/μg).

Oligomeric forms of EGFR were stabilized and demonstrated as follows: A-431 cells were grown to a monolayer, washed with BS medium (RPMI with 0.1% BSA, 20 mM HEPES, 2.4 mg/ml glucose), and kept for 40 min in the same medium, containing 500 nM 12-0-tetradecanoylphorbol-13-acetate (TPA) or 1 mM ATP (or without additives), after which the cells were incubated for 30 min at 37°C in the presence of ^{125}I-EGF; the unbound ligand was removed by washing 3 times with BS, and cross-linkers were added: 15 mM 1-ethyl-3-[3(dimethylamino)propylcarbodiimide (EDAC) (Pierce, The Netherlands) or 0.5 mM bissuccinylimidyl suberate (DSS) (Sigma, USA), for 15 min at 37°C and at 4°C respectively; the cells were then washed with BS and solubilized in buffer for samples according to Laemmli [8].

To study dimers of EGF receptors by the autophosphorylation reaction, A-431 cells grown on 24-well plates to the monolayer state were incubated with TPA or with BS medium, EDF was added to a concentration of 200 nM for 30 min at 37°C, after which the sample was washed with BS and treated with cross-linking agents and solubilized in buffer containing 1% Triton X-100, 20 mM HEPES, pH 7.4, for 1 h at 4°C; the unsolubilized material was sedimented by centrifugation. Aliquots of supernatant and residue (10 μl) were incubated with EGF (200 nM) for 1 h at 4°C, and the phosphorylation reaction was carried out as described previously [2].

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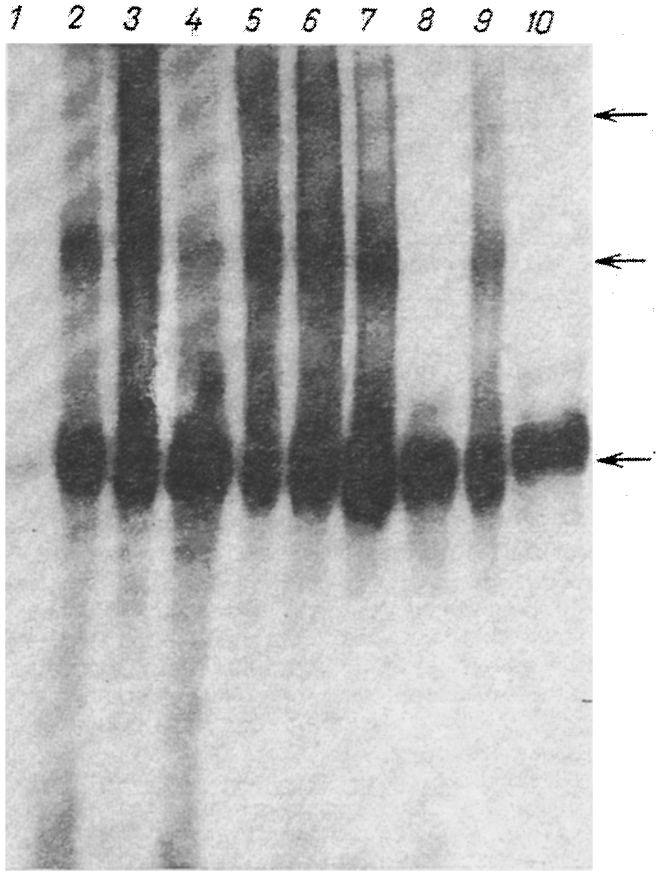

Fig. 1. Covalent labeling of A-431 cells with ^{125}I-EGF. Autoradiograph of gradient (3-10%) PAG. Cells were kept at 37°C (1-9) or at 4°C (10) in the presence of TPA (3, 6), of ATP (4, 7), or without additives (1, 2, 5, 8, 9, 10), and incubated with ^{125}I-EGF, without (2-10) or with (1) a 500-fold excess of EGF, and treated with cross-linkers: EDAC (2, 3, 4) or DSS (1, 5, 6, 7, 9, 10), and lyzed in buffer for samples. Levels of 170, 350, and 530 kD are indicated by arrows.

Electrophoretic separation of the proteins was carried out in PAG with SDS in a Laemmli buffer system [8]; after electrophoresis the gels were stained, dried in vacuo, and exposed with x-ray film for 72 h.

EXPERIMENTAL RESULTS

Initially we verified the possibility of identifying EGFR dimers. A-431 cells were incubated in the presence of 200 nM ^{125}I-EGF, and then treated with cross-linking agents. The results showed that the use of DSS stabilizes both ligand—receptor and receptor—receptor bonds much more effectively.

Thanks to the use of ^{125}I-EGF with high specific activity (over 200,000 cpm) and electrophoresis on 3-10% gradient gels, enabling the proteins to be fractionated within the range from 30 to 650 kD, not only dimers but also trimers of EGFR could be detected (Fig. 1). The latter had a molecular weight close to that expected. namely about 550 kD ($170 \times 3 + 6 \times 3$). Incidentally, the efficacy of detection of trimers was significantly higher if DSS rather than EDAC was used as the cross-linking agent. Meanwhile, in cells treated with EDAC, a protein labeled with ^{125}I-EGF and with mol. wt. of about 250 kD was clearly found. The nature of this protein is not clear: either it is an EGF receptor, which has bound labeled EGF and a certain substrate, or it is a special EGF-binding protein. It was also found when DSS was used, but much less distinctly.

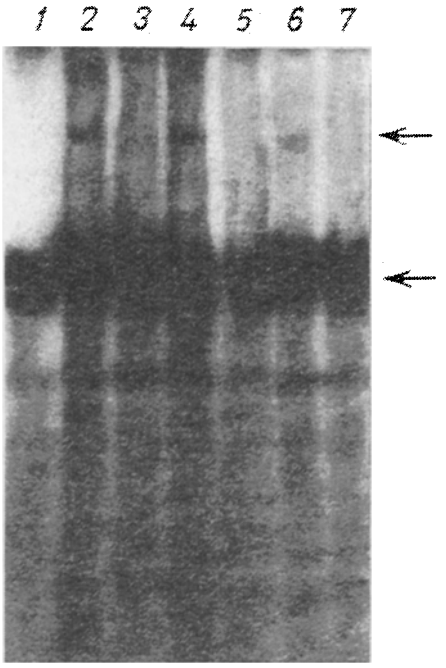

Fig. 2. Phosphorylation on solubilized A-431 cells. Cells kept for 40 min in presence of TPA (6, 7) or without additives (1-5), were incubated with 200 nM EGF (1, 2, 4, 6) or without EGF (3, 5, 7), treated with DSS (2, 3, 6, 7) and EDAC (4, 5) or not treated with cross-linkers (1); cells were then solubilized, reincubated with 200 nM EGF, and subjected to the phosphorylation reaction. Arrows indicate levels of 170 and 350 kD.

Incubation of the cells with TPA leads to disappearance of high-affinity EGF-R [4]. Meanwhile, when treatment with TPA was followed by covalent labeling with ^{125}I-EGF, the content of dimers was unchanged (Fig 1). As recent investigations [7] have shown, extracellular ATP stimulates the accumulation of inositol-1,4,5-triphosphate in A-431 cells and raises the Ca^{2+} level. Under these circumstances activation of protein kinase C takes place, and this is evi responsible for the decrease in affinity of the EGF receptors after treatment of the cells with ATP. It will be clear fr 1 that pretreatment of the cells with ATP also had virtually no effect on the concentration of dimers of the receptors. This indicates that the two classes of EGFR may participate in dimer formation, or, as is found more frequently, low-affinity receptors may undergo oligomerization.

Similar results were obtained in a study of the effect of TPA on phosphorylation of EGFR; treatment of the cells with phorbol ester did not prevent dimer formation (Fig. 2). Incidentally, according to our findings, stabilization of phosphorylated EGFR by cross-linkers is much less effective than after covalent labeling with ^{125}I-EGF. The reason may perhaps be that dimer formation is the stage preceding auto- and interreceptor phosphorylation, but phosphorylated receptors may have weaker affinity for one another. In an attempt to carry out the autophosphorylation reaction and then to stabilize dimers by a cross-linker, a sharp fall was observed in the intensity of phosphorylation, evidently due to a disturbance of the conformation of EGFR after treatment with cross-linking agents. The efficacy of oligomerization of EGFR was tested at 37°C and at 4°C. It was found that although at 4°C monomers of the receptor are sufficiently intensively labeled by ^{125}I-EGF, no labeled dimers were found (Fig. 1). Meanwhile, incubation of cells with the corresponding ligand at 4°C enables the kinase of their receptors to be effectively activated [11, 12]. Consequently, activation of EGFR can take place in principle without the oligomerization stage, or the number of receptors activated by oligomerization cannot be detected by the methods used.

In order to discover whether interaction with EGF is an essential condition for oligomerization, we fixed A-431 cells with 10% formalin, thereby preventing displacement of the receptor molecules in the plane of the membrane, after which covalent labeling was carried out with ^{125}I-EGF. It will be clear from Fig. 3 that the intensity of the signal at the 340 kD level was almost indistinguishable in intact cells and cells pretreated with formalin. The results are in good agreement with recently published data indicating that receptor oligomers can exist on membranes even in the absence of binding of

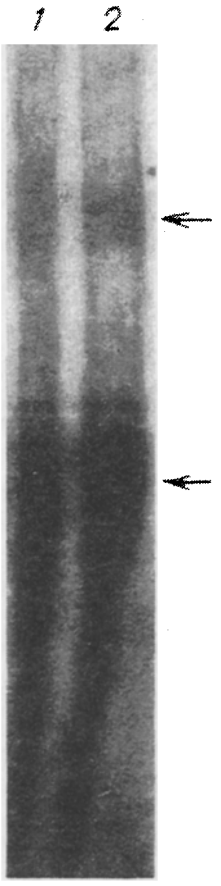

Fig. 3. Covalent labeling by ^{125}I-EGF of A-431 cells previously fixed with formalin (1) or intact (2). Arrows indicate 170 and 350 kD levels.

the ligand. It can be tentatively suggested on the basis of these results that the formation of EGFR oligomers is not directly connected with a change in their affinity, that some receptors are dimerized in the absence of the ligand, and addition of the ligand to the receptor stimulates the formation not only of dimers, but also of trimers and, perhaps, of complexes with other proteins also.

The results are evidence that not all the features of ligand-receptor interaction and stimulation of the kinase activity of the EGF receptor can be explained by means of the intermolecular activation model. Further research is necessary in order to study interreceptor interactions at the level of different domains: ligand-binding, transmembrane, and cytoplasmic (kinase), in order to decipher the complex mechanisms of reactions of generation and transmission of the mitogenic signal.

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