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Epidermal growth factor induces tyrosine phosphorylation of epidermal growth factor receptors not occupied with ligand in intact A431 cells

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The mechanism of epidermal growth factor receptor (EGF-R) autophosphorylation in intact A431 cells was studied. We detected epidermal growth factor (EGF) induced tyrosine phosphorylation of EGF-R not occupied with ligand. Cell monolayers were subjected to irradiation after incubation with photoreactive derivative of EGF and uncoupled EGF was extracted by acidic treatment. Subsequent immunoprecipitation with antiphosphotyrosine antibodies resulted in precipitation of both EGF-R complexes with EGF and EGF-R with unoccupied ligand-binding site. The fact of precipitation of EGF-R with unoccupied ligand-binding site in conjunction with our finding of rapid dephosphorylation of EGF-R after EGF extraction by acidic treatment, strongly supports the interpretation that cross-phosphorylation of EGF-R may take place in intact cells.

Epidermal growth factor receptor; Autophosphorylation; Antiphosphotyrosine antibody; A431 cell

1. INTRODUCTION

Ligand-induced autophosphorylation of EGF-R is thought to be a crucial step in the process of receptor-mediated signalling. Receptor autophosphorylation may proceed by two distinct mechanisms: by an intramolecular or by an intermolecular mechanism [1]. In an intramolecular process the phosphorylation sites of receptor are phosphorylated by the protein kinase part of the same EGF-R molecules. Alternatively, one receptor may function as a substrate for the tyrosine kinase of the other (intermolecular mechanism). To date, no evidence on intermolecular cross-phosphorylation of native EGF-R in intact cells has been obtained.

This paper reports EGF inducing of the tyrosine phosphorylation of EGF-R not occupied with ligand in intact A431 cells (the most common object for study of EGF-R function [2]). These data are in correspondence with intermolecular model of EGF-R autophosphorylation.

2. MATERIALS AND METHODS

EGF was purified from mouse submaxillary glands as described in [3] and iodinated using Iodo-Gen (Serva). Human epidermoid carcinoma A431 cells were obtained from the Cell Culture Collection (Institute of Cytology, USSR) and maintained as in [4]. The preparation of antiserum against phosphotyrosine (anti-P-Tyr) has been described previously [5]. EGF and [^{125}I]EGF were coupled to *N*-5-azido-2-nitrobenzoyloxysuccinimide (ANB-NOS) as follows. To 100 μl of dimethyl sulfoxide (Fluka) was added 150 μg of ANB-NOS (Sigma). 10 μl of this solution was added to 0.5 μg EGF or [^{125}I]EGF dissolved in 100 μl of sodium phosphate buffer (100 mM, pH 7.6). The coupling was allowed to proceed overnight and was stopped by adding 30 μl of lysine dissolved in H_2O (100 mM).

For EGF-induced autophosphorylation experiments cells were plated on 24-well plastic plates and used 2-days after plating. Cells were incubated in the dark with photoreactive EGF in medium, containing MEM, 0.1% BSA (Serva), 20 mM Hepes (pH 7.2), for 1-2 h at 4°C (Fig. 1a) and then were subjected to irradiation for 10 min with UV viewing light at a height of 8 cm above the surface of the monolayer (Fig. 1b). The irradiation was conducted at 4°C and the light was filtered through a filter with maximum of omission 340 nM. The efficiency of cross-linking was about 12-20% as determined from the decrease in extracted by mild acidic treatment of ^{125}I]EGF-ANB-NOS in irradiated cells. After rinsing with cold PBS cells were treated twice for 3 min with acetate buffer solution (pH 4.5), containing 0.05 M sodium acetate buffer, 150 mM NaCl (ABS) at 4°C. Such acidic treatment allows extraction of about 95% of the total surface-bound [^{125}I]EGF (not shown) without removal of the covalent complex EGF-receptor (Fig. 1c). After acidic treatment cells were washed 3 times with cold PBS and were lyzed in RIPA buffer, containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% deoxycholate, 1% NP-40, 1 mM EDTA, 0.1% NaN_3 and 5 mM Na orthovanadate. Lysates were incubated for 30 min at 4°C with fixed (*Staphylococcus aureus* previously incubated with pre-immune antiserum. Lysates were cleaved by centrifugation and anti-P-Tyr were added for incubation of 30 min at 4°C (Fig. 1d). Immunocomplexes were collected using fixed *Staphylococcus aureus* (Fig. 1e), washed 3 times with RIPA buffer. To detect the amount of EGF-binding sites in immunoprecipitate the last washing during immunoprecipitation was performed in RIPA buffer with Tris-HCl replaced by 20 mM Hepes. Immunoprecipiated EGF-R were incubated with ^{125}I]EGF-ANB-NOS in the dark for 1 h (4°C) and cross-linking was performed with UV viewing light (Fig. 1f). SDS-PAGE was performed as described by Laemmli [6]. For metabolic labelling of cell proteins, the cultures were incubated overnight either in methionine-free DMEM containing [^{35}S]methionine

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Abbreviations: EGF, epidermal growth factor; EGF-R, epidermal growth factor receptors; PBS, phosphate-buffered saline; anti-P-Tyr, antiserum against phosphotyrosine

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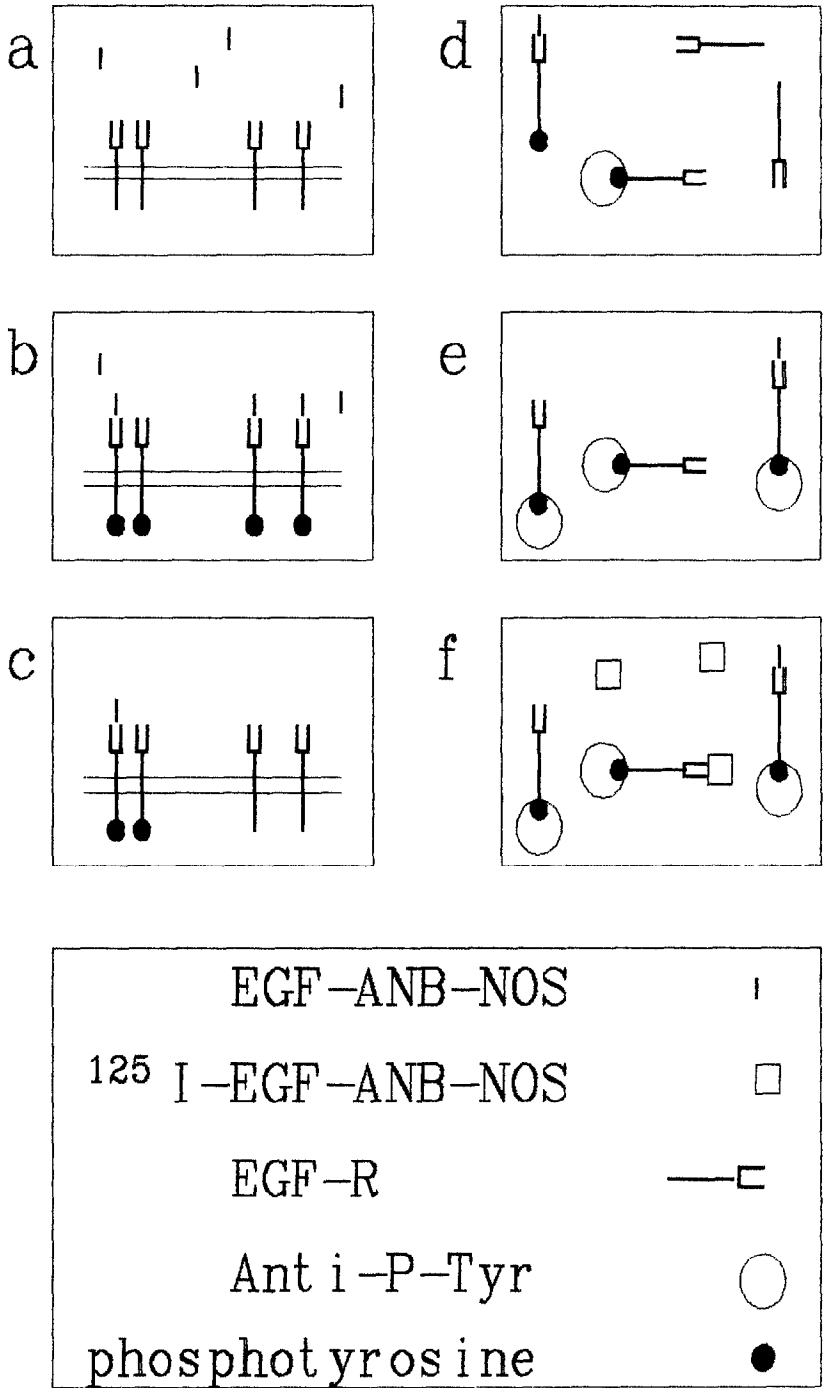

Fig. 1. Schematic description of the protocol of the experiment used to determine EGF-induced increase of phosphotyrosinilated receptors not occupied with ligand.

(100 μCi/ml), or in phosphate-free DMEM containing [^{32}P]phosphate (100 μCi/ml).

3. RESULTS AND DISCUSSION

Intermolecular mechanism of autophosphorylation of EGF-R must result in possible appearance of phosphotyrosinilated EGF-R not occupied with ligand. On the contrary, intramolecular mechanism of autophosphorylation permits only complexes of ligand-receptor to be phosphorylated on tyrosine. The scheme of the experimental procedures used for the revealing of phosphotyrosinilated EGF-R with free ligand-binding site is shown in Fig. 1.

In our experiments the cells incubated with EGF-ANB-NOS at 4°C were exposed to UV viewing light

followed by removal of the non-cross-linked EGF-ANB-NOS by mild acidic treatment. The ability of non-cross-linked photoreactive EGF to be removed by mild acidic treatment was shown previously [7]. Cells were lyzed and after immunoprecipitation with anti-P-Tyr the existence of EGF-binding sites was checked: immunoprecipitated material was incubated with [^{125}I]EGF-ANB-NOS followed by exposition to UV viewing light. After cross-linking, proteins were separated by electrophoresis. While no protein was cross-linked with [^{125}I]EGF-ANB-NOS in immunoprecipitated material from untreated cells, the protein with apparent M_r of 170 000 was strongly labeled in EGF-ANB-NOS treated A431 cells (Fig. 2). Covalent complexes of EGF-ANB-NOS-receptor immunoprecipitated with anti-P-Tyr could not be detected with [^{125}I]EGF-ANB-NOS, because their ligand-binding sites have been already occupied. Unoccupied phosphotyrosinilated receptors immunoprecipitated with anti-P-Tyr were able to be cross-linked with [^{125}]EGF-ANB-NOS (Fig. 1). The amount of onoccupied EGF-R phosphorylated on tyrosine depended on the concentration of unlabeled EGF-ANB-NOS used for A431 cells treatment (Fig. 2a,b,c,d). Immunoprecipitation was completely inhibited by excess (2 mM) of free phosphotyrosine (Fig. 2e).

Immunoprecipitation with polyclonal antibodies to EGF-R was used as a positive control (Fig. 2f). Immunoprecipitation with polyclonal antibodies to EGF resulted in precipitation of covalent complexes of EGF-ANB-NOS with receptor with no free sites for cross-linking of [^{125}I]EGF-ANB-NOS, so it was used as a negative control (not shown).

To analyze the data obtained, an important question arises whether the receptor is rapidly dephosphorylated after removal of EGF by mild acidic treatment. In order to test the extent of EGF-R autophosphorylation after EGF removal we have utilized cells labeled overnight with [^{32}P]orthophosphate. Anti-P-Tyr did not precipitate any labeled receptor immediately after EGF

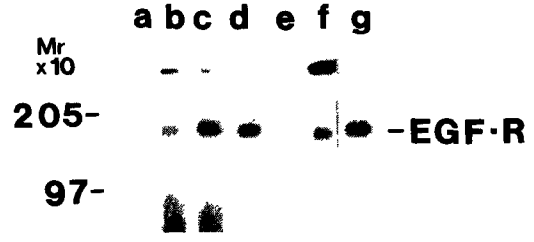

Fig. 2. Effect of EGF-ANB-NOS concentration on the amount of EGF-R phosphorylated on tyrosine with unoccupied [^{125}I]EGF-ANB-NOS binding site. A431 cells were incubated either in the absence of EGF-ANB-NOS (a), or in the presence of 10 ng/ml (b), 100 ng/ml (c,e,f,g), 200 ng/ml (d) of EGF-ANB-NOS. After treatment with ABS the lysis was performed either immediately (a,b,c,d,f) or after 20 min of incubation in PBS (g). The precipitation was performed either with anti-P-Tyr antibodies (a,b,c,d,e,g) or with polyclonal antibodies against EGF-R (f).

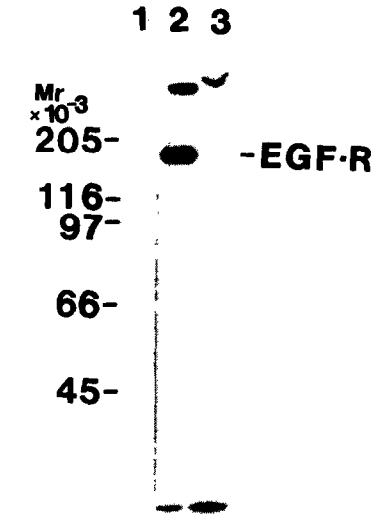

Fig. 3. Effect of the removal of EGF on the dephosphorylation of EGF-R. A431 cells labelled with [^{132}I]phosphate were untreated (1) or incubated with EGF without (2) or with (3) subsequent treatment with ABS. Phosphoproteins were immunoprecipitated with anti-P-Tyr antibodies.

removal (Fig. 3). Thus, the removing of non-cross-linked EGF-ANB-NOS by acidic treatment caused rapid dephosphorylation of previously autophosphorylated receptors and the appearance of receptors containing phosphotyrosine was a result of the formation of covalent complex of EGF-ANB-NOS and receptor. This conclusion was also supported by the fact that the amount of unoccupied EGF-R containing phosphotyrosine did not diminish, when the time period between acidic treatment and cell lysis was in-

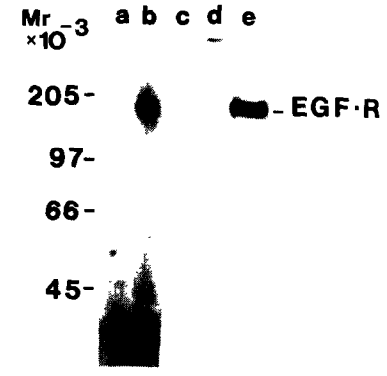

Fig. 4. The absence of co-precipitation of ^{35}S-labelled unphosphorylated EGF-R during the precipitation with anti-P-Tyr. Whole cell solubilized material metabolically labelled with [^{35}S]methionine was added (c,d) or not (a,b) during solubilization to the cell culture preincubated (b,d) or not (a,c) with EGF-ANB-NOS. The ability of EGF-ANB-NOS to induce the appearance of phosphotyrosinilated receptors with unoccupied ligand binding site was checked on parallel cultures by cross-linking with [^{125}I]EGF-ANB-NOS (a,b). The presence of metabolically labelled EGF-R in solubilized material was proved by precipitation with anti-P-Tyr after EGF treatment of [^{35}S-labelled cells (e).

creased (Fig. 2g). Recently the rapid dephosphorylation of phosphorylated receptor molecules upon removal of EGF from binding sites by a short acid wash was shown [8].

We suppose that after removing of non-cross-linked EGF-ANB-NOS the appearance of phosphotyrosinilated receptors with unoccupied free ligand-binding site might be the result of tyrosine kinase activity of covalent complexes of EGF-ANB-NOS with EGF-R. An ability of cross-linked proteins to retain enzyme activity was shown [9].

In contrast to EGF-ANB-NOS, EGF in the same concentrations and experimental conditions (binding, irradiation, acidic treatment) failed to produce unoccupied EGF-R containing phosphotyrosine (not shown). In this case all EGF molecules were non-cross-linked and the absence of unoccupied phosphotyrosinilated EGF-R detected by [^{125}I]EGF-ANB-NOS after immunoprecipitation with anti-P-Tyr proved that non-cross-linked EGF-ANB-NOS were not responsible for appearance of free EGF-R containing phosphotyrosine. This is consistent with the notion that ligand-receptor complexes were responsible for phosphorylation of unoccupied receptors.

It remained possible that unphosphorylated receptors were co-precipitated with phosphorylated receptors by anti-P-Tyr, because of the process of receptor dimerization. However, it was shown that EGF-R dimers dissociate in the presence of 0.5% sodium deoxycholate [10]. We used RIPA buffer, containing 1% sodium deoxycholate, and also tested the ability of anti-P-Tyr to co-precipitate unphosphorylated receptors labelled with [^{35}S]methionine (Fig. 4). Metabolically labelled cell material was previously devoid of phosphorylated EGF-R by immunoprecipitation. No co-precipitation of ^{35}S-labelled unphosphorylated receptors with receptors from EGF-ANB-NOS treated cells was detected (Fig. 4c,d). To examine whether metabolically labelled EGF-R were present in solubilized material used to determine possible co-precipitation, parallel cell cultures were treated with EGF and then the lysate was immunoprecipitated with anti-P-Tyr (Fig. 4e). It also may be concluded from the results presented in Fig. 4, that the phosphorylation of onoccupied EGF-R takes place at the cell level. Recently it was shown that in solution the mutated forms of EGF receptors participate in the intermolecular process of autophosphorylation [11]. The data reported here indicate that in intact cells the EGF-induced appearance of phosphotyrosinilated unoccupied native EGF receptors may take place.

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