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Functional State of the Epidermal Growth Factor-Receptor Complexes during Their Internalization in A-431 Cells

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Functional state of internalized epidermal growth factor (EGF) receptor in A-431 cells has been studied. The use of photoaffinity [^{125}I]EGF derivative allowed us to establish that inside the cell the EGF retains its connection with the receptor. With the help of polyclonal antibodies to phosphotyrosine, it has been shown that EGF-receptor complexes maintain their phosphorylated state during internalization. The internalized EGF receptor kinase as well as that localized in the plasma membrane appeared to be able to phosphorylate synthetic peptide substrate introduced into the cell.

The epidermal growth factor (EGF) receptor is a 170-kilodalton (kDa) integral membrane glycoprotein containing cytosol-oriented tyrosine-specific kinase (for a review, see reference 18). EGF binding stimulates receptor tyrosine kinase, resulting in both receptor and substrate phosphorylation (for a review, see reference 10). There is a lot of data showing that receptor kinase activity plays an important role in the transmission of mitogenic signal (for a review, see references 20 and 25).

After EGF binding, the ligand-receptor complexes are rapidly internalized; they are then delivered to various endosomal structures and, finally, degraded within lysosomes (8, 9, 12, 21, 22). The role of the internalization process in triggering the biochemical reactions leading to DNA synthesis so far remains obscure. To elucidate this question, the functional state of internalized EGF receptor should be determined.

Whether the EGF receptors retain their functional activity during the translocation from plasma membrane into endosomal compartment is yet to be clarified. Thus, the presence of active EGF receptor in the fractions of intracellular components isolated from homogenates of A-431 cells (5) and rat liver cells (13) has been shown. On the other hand, from the results of experiments on intact A-431 cells, Sturani and co-workers concluded that internalized EGF receptors failed to maintain the phosphorylated state of tyrosine residues (24). In this study, we compared the functional state of internalized and membrane-localized EGF-receptor complexes by using two experimental approaches: (i) cell exposure to EGF at low temperature, which is known to completely block internalization (1) and (ii) removal of surface-bound EGF by cell treatment with weak acid buffer (50 mM sodium acetate [pH 4.5 to 5.0] containing 150 mM NaCl). To identify phosphotyrosine-containing proteins, we have used affinity-purified polyclonal antibody to phosphotyrosine (anti-P-Tyr). As has been established previously, the major phosphoprotein interacting with this antibody in the EGF-stimulated A-431 cells is the EGF receptor (17).

Effect of mild acid wash on EGF receptor Tyr phosphorylation. In two parallel series of experiments, the cells were preincubated with EGF at 4°C and then placed at 37 or 4°C for an hour. During this period of incubation at 37°C, from 60 to 75% of cell-associated [^{125}I]EGF was found to be internalized (data not shown). After the incubation with EGF, some of the cultures were subjected to acid treatment. In the first series of experiments (Fig. 1), the cells were metabolically labeled with [^{32}P]orthophosphate and Tyr-phosphorylated receptors were detected by immunoprecipitation with anti-P-Tyr, while in the second series of experiments the receptors were detected by immunofluorescence staining (Fig. 2). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of immunoprecipitated proteins has shown that at 4°C, EGF markedly stimulates Tyr phosphorylation of a 170-kDa protein corresponding to the known molecular mass of EGF receptor (2). Under such conditions, immunostaining is observed along the cell margins, which is a typical pattern for the distribution of membrane receptors (8, 21, 22). Removal of the surface-bound EGF by acid treatment of the cells incubated with EGF at 4°C resulted in rapid receptor dephosphorylation. In contrast, in the case of incubation of cells at 37°C, when internalization took place, such acid treatment did not result in any significant decrement of phosphotyrosine content in the receptor molecule, which indicates that during internalization the receptors remain Tyr phosphorylated. Immunofluorescence analysis shows that inside the cell, phosphorylated receptors are mainly concentrated in the juxtanuclear area which has earlier been identified as the para-Golgi region (22).

Photoaffinity labeling of the EGF receptor. To prepare the photoaffinity EGF derivative, [^{125}I]EGF was coupled to the heterobifunctional reagent *N*-5-azido-2-nitrobenzoyloxysuccinimide (Sigma Chemical Co.) as described by Hock and co-workers (11). The cells were allowed to internalize photoaffinity [^{125}I]EGF, and then some of the cultures were subjected to acid treatment. At the end of the procedure described in detail in the legend to Fig. 3, the cells were UV irradiated to provoke [^{125}I]EGF-receptor covalent coupling. After that, the cells were solubilized and two equivalent portions of each extract were analyzed. One of them was immunoprecipitated with anti-P-Tyr, and then both the immunoprecipitate and the total extract were processed by electrophoresis. Radioactivity corresponding to 175-kDa bands and total cell-associated radioactivity were measured. The immunoprecipitation efficiency determined by the additional immunoprecipitation from the same extract with a new portion of anti-P-Tyr appeared to be equal to 90% (data not shown). In control experiments, when the internalization was blocked by low temperature (Fig. 3, lanes a), the acid treatment resulted in a strong decrease in the radioactivity of

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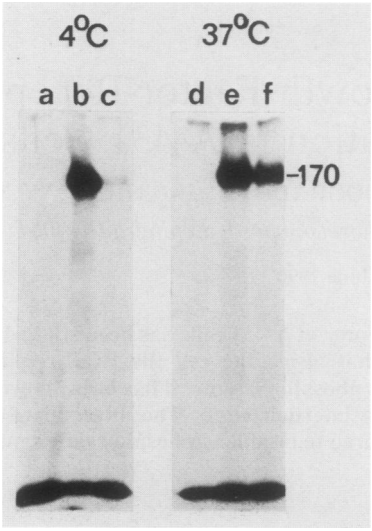

FIG. 1. Effect of mild acid wash on the phosphotyrosine content in internalized EGF receptors and those located in the plasma membrane. Cells were labeled with [^{32}P]orthophosphate and exposed to 200 ng of EGF per ml (lanes b, c, e, and f) or EGF-free medium (lanes a and d) for an hour at 4°C. Then, some of the cultures in the same medium were placed at 37°C for an hour (lanes d, e, and f), while the others were left at 4°C (lanes a, b, and c). After incubation, the cells were either immediately extracted (lanes a, b, d, and e) or washed with the acid (lanes c and f). Phosphotyrosine-containing proteins were immunoprecipitated with 20 μg of anti-P-Tyr, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and identified by autoradiography. Molecular size, in kilodaltons, is shown on the right.

175-kDa bands. The data suggest that lack of sensitivity of photoaffinity [^{125}I]EGF to acid treatment indicates its intracellular location and is not due to spontaneous covalent ligand-receptor coupling.

Using photoaffinity [^{125}I]EGF derivative, we analyzed EGF-receptor interactions directly within endosomes. Comparison of lanes b and c in Fig. 3 shows that acid treatment results in the same decrease in the radioactivity of both 175-kDa bands and in the total cell-associated radioactivity. These results show that the photoaffinity [^{125}I]EGF attach to the receptors in endosomes as effectively as in plasma membrane, which indicates that during internalization no significant dissociation of ligand-receptor complexes occurs. The data obtained are in agreement with electron microscopy observations (3, 16) and our previous results on cells permeabilized with detergent (21). It should be noted that the slow degradation kinetics of EGF-receptor complexes is a peculiarity of A-431 cells (15). For instance, in human diploid fibroblasts, receptor degradation is much more rapid (23).

The immunoprecipitation of covalent [^{125}I]EGF-receptor complexes has shown that only about 15% of them are capable of interacting with anti-P-Tyr, which points to functional heterogeneity of the EGF receptor population in A-431 cells. King and Cooper (14), as well as Sturani and co-workers (24), came to the same conclusion by using other experimental approaches. As has been found previously for the insulin receptor (19), it can be assumed that there are at least two EGF receptor subpopulations which differ in phosphorylation state. The fact that the percentage of tyrosine-phosphorylated receptors remains unchanged during internalization shows that inside the cell, the receptors are not affected by dephosphorylation.

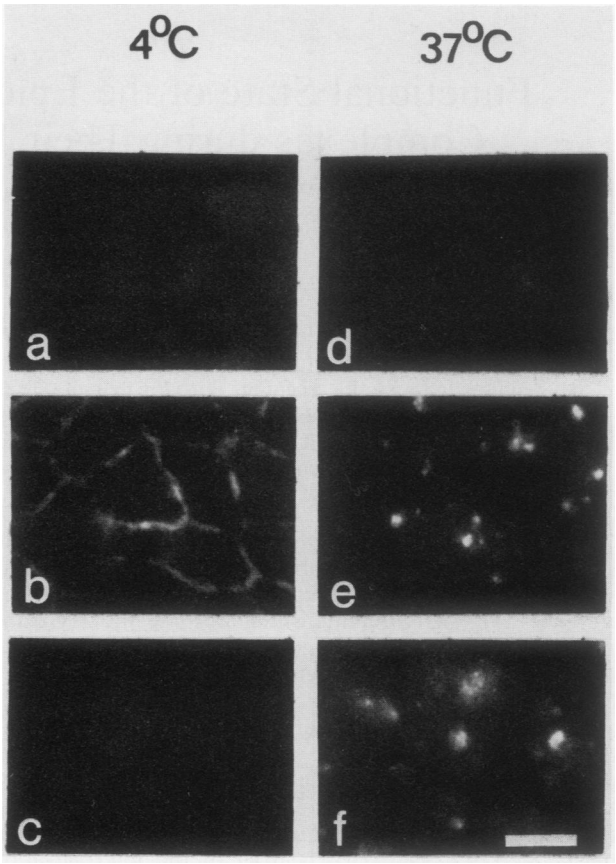

FIG. 2. Immunofluorescent staining of A-431 cells with anti-P-Tyr. Cells on cover slips were incubated with EGF and washed as described in the legend to Fig. 1. At the end of the procedure, cells were fixed with 4% formaldehyde, permeabilized with 0.1% Triton X-100, and stained with anti-P-Tyr. The second antibodies used were fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G. Lanes in Fig. 1 correspond to the panels in Fig. 2. Bar, 50 μm.

Evaluation of receptor kinase activity. In the experiments on the intact cells, the protein kinase activity of internalized EGF receptor was indirectly estimated by the phosphotyrosine content in the receptor molecule (3, 24). However, the amount of phosphorylated receptors depends on at least two additional factors which can vary during the internalization: (i) the tyrosine phosphatase activity in the cell interior and (ii) the competition for tyrosine kinase between different intracellular substrates, on one side, and receptor autophosphorylation sites, on the other. Consequently, the direct method should be employed to evaluate the activity of the EGF receptor tyrosine kinase during endocytosis. For this purpose, the exogenous tyrosine kinase substrate and [γ-^{32}P]ATP were introduced by mild digitonine permeabilization into the cells treated as described in the legend to Fig. 4. After the cells were treated, the EGF-stimulated tyrosine kinase activity was assayed. The reaction was carried out as described by Giugni and co-workers (7), except that the reaction mixture was supplemented with synthetic peptide. Briefly, the phosphorylation reaction was initiated by the addition of reaction mixture containing 50 μM [γ-^{32}P]ATP, the synthetic peptide substrate for tyrosine kinase (1 mg/ml), and 0.01% digitonine. Phosphorylation was allowed to proceed for 10 min at 4°C, after which the reaction was terminated by the addition of an equal volume of a double

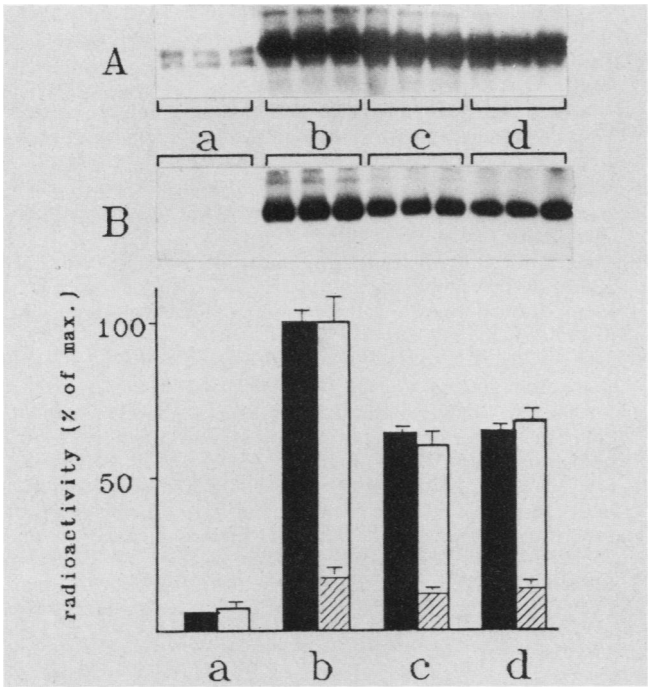

FIG. 3. Photoaffinity labeling of EGF receptor. The cells were preexposed to photoaffinity [^{125}I]EGF (200 ng/ml) at 4°C and then placed at 37°C for an hour (lanes b, c, and d), while the control cultures (lanes a) were left at 4°C. At the end of incubation, the cells in lanes a, c, and d were treated with the acid. Then, the cells were extensively washed and UV irradiated either immediately (lanes a, b, and c) or after being incubated in EGF-free medium for an hour at 37°C (lanes d). The irradiation was performed at 4°C with a UV lamp equipped with a 340-nm glass filter for 10 min. Prior to the UV irradiation, all the procedures were carried out under dim red light. Then, the cells were lysed and two 40 μl portions of each extract were analyzed. One of them was loaded directly on a sodium dodecyl sulfate-polyacrylamide gel (A), and the remainder was immunoprecipitated with anti-P-Tyr (B). The gels were subjected to autoradiography; after that, the 175-kDa bands were cut out of the gel and counted. Only the upper parts of the gels are shown. Panel B is taken from a longer exposure than panel A. ▮, Total cell-associated radioactivity; □, radioactivity corresponding to 175 kDa bands; ▨, relative amount of immunoprecipitated 175-kDa complexes. Error bars indicate standard deviations of the mean.

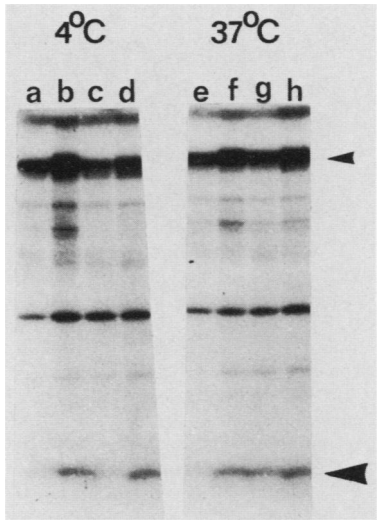

FIG. 4. Effect of mild acid wash on the kinase activity of the internalized EGF receptors and those located in the plasma membrane. The cells were preexposed to 200 ng of EGF per ml (lanes b, c, d, f, g, and h) or the EGF-free medium (lanes a and e) at 4°C and then either kept at low temperature (lanes a, b, c, and d) or placed into 37°C for an hour (lanes e, f, g, and h). After incubation, some of the cultures (lanes c, d, g, and h) were washed with acid. Then two acid-washed plates were subjected to the additional incubation for an hour either in the cold (4°C) medium supplemented with 200 ng of EGF per ml (lane d) or in the warm (37°C) EGF-free medium (lane h). The top and the bottom arrows indicate the positions of the EGF receptor and the exogenous peptide substrate, respectively.

concentration of sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer. The samples were loaded onto a gel with an 8 to 30% polyacrylamide gradient and analyzed by electrophoresis. To facilitate the detection of Tyr-phosphorylated proteins, the gel was then treated with 1 N KOH (6). For the exogenous substrate, we used synthetic peptide with a phosphorylation site similar to the viral tyrosine kinase pp60^{src} (a kind gift of C.-H. Heldin, Ludwig Institute for Cancer Research, Uppsala Branch). The peptide contains tyrosine as the single amino acid that can be phosphorylated (4).

The results presented in Fig. 4A show that the removal of EGF from membrane receptors completely switches off their tyrosine kinase activity toward peptide substrate. The reexposition of the acid-washed cells to EGF restored the enhanced substrate phosphorylation, which indicates that the loss of tyrosine kinase activity is not an artifact of the acid wash. In contrast, when cells were allowed to internalize EGF (Fig. 4B), the acid treatment had no significant effect on the ability of cells to phosphorylate the substrate. Moreover, an elevated level of substrate phosphorylation was maintained during further incubation of acid-washed cells at 37°C.

All the data presented here demonstrate that the EGF receptors are internalized in their activated state and retain tyrosine kinase activity for at least an hour after internalization.

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