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Demonstration of Epidermal Growth Factor-induced Receptor Dimerization in Living Cells Using a Chemical Covalent Cross-linking Agent*

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We have used the soluble covalent cross-linking agent 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDAC) to examine the capacity of epidermal growth factor (EGF) to stimulate the dimerization of purified EGF receptor, of EGF receptor in membrane preparations and in intact A431 cells. The addition of EGF either to membranes from A431 cells or to EGF receptor which was purified from A431 cells by immunoaffinity chromatography caused the appearance of a cross-linked product of M_r 340,000 which was identified using EGF receptor-specific antibodies as an EGF receptor dimer. Three independent approaches including biosynthetic labeling, surface iodination, and immunoblotting experiments were utilized to follow EGF receptor dimerization in living A431 cells. These approaches provided consistent results indicating that EGF induced rapid dimerization of EGF receptor in living cells, suggesting that this process may play a role in transmembrane signalling mediated by EGF.

Epidermal growth factor (EGF)[1] acts by binding to and activating a membrane glycoprotein denoted as the EGF receptor (EGF-R) (reviewed in Refs. 1–3). The EGF-R is a single chain polypeptide of M_r of 170,000 composed of three major domains: a large extracellular ligand binding domain, a single hydrophobic membrane spanning region, and a cytoplasmic region with intrinsic protein tyrosine kinase activity (4). The binding of EGF to the extracellular ligand-binding domain activates the cytoplasmic kinase domain which undergoes self-phosphorylation and subsequently phosphorylates various cellular substrates (5, 6). Numerous studies have shown that EGF can stimulate the protein tyrosine kinase activity of its receptor molecule without the involvement of additional molecules (7, 8). Hence, an interesting and unresolved question is the mechanism of signal transduction between the hormone ligand-binding domain to the cytoplasmic domain leading to the stimulation of the protein tyrosine kinase function (3). Yarden and Schlessinger (3, 8, 9) have demonstrated that purified EGF receptor undergoes rapid, reversible EGF-induced oligomerization and that receptor oligomerization is an intrinsic property of the EGF-R. Several lines of evidence support the notion that receptor oligomerization may have an important functional role. First, EGF-induced receptor self-phosphorylation has a parabolic dependence on the concentration of EGF-R, suggesting an intermolecular step for the activation of the kinase function (8). Second, cross-linking of purified EGF-R by antibodies or lectins stimulates receptor self-phosphorylation. Third, immobilization of EGF-R on various solid matrices prevents EGF from activating the kinase function; and fourth, cross-linking of EGF-R increases their affinity towards EGF. On the basis of these results, an allosteric aggregation model was formulated for the activation of the kinase function of the EGF-R by EGF (3, 8). According to this model, EGF induces receptor oligomerization which brings together adjacent cytoplasmic tyrosine kinase domains leading to their activation by receptor-receptor interactions. Such a mechanism will bypass the requirement for an energetically unfavorable conformational change to be transferred through the transmembrane region connecting the two functional domains, an essential requirement of an intramolecular model for receptor activation (10).

In this report, we describe experiments utilizing a soluble covalent cross-linking agent to demonstrate that EGF induces receptor dimerization. We show that both purified EGF-R and the EGF-R in membrane preparations from A431 cells undergo ligand-induced receptor dimerization. Moreover, a similar dimerization of EGF-R occurs upon the addition of EGF to intact A431 cells indicating that ligand-induced receptor dimerization exists also *in vivo*, thus suggesting that this process may be relevant to transmembrane signalling mediated by EGF.

EXPERIMENTAL PROCEDURES

Materials—[γ-^{32}P]ATP (3,000 Ci/mmol) was purchased from the Radiochemical Centre, Amersham. EGF was supplied by I.D.L. (Jerusalem) and radiolabeled with Na^{125}I by the lactoperoxidase method. Molecular weight standards for gel electrophoresis were from Bio-Rad. Wheat germ agglutinin and *Lens culinaris* hemagglutinin were obtained from Bio-Yeda (Rehovot). Protein A coupled to Sepharose was obtained from Pharmacia LKB Biotechnology Inc. All other reagents were from Sigma.

Cells—Human epidermoid carcinoma cells (A431) and mammary carcinoma cells (MDA-MB468) were cultured in Dulbecco's modified

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[1] The abbreviations used are: EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; MES, 4-morpholineethanesulfonic acid; DMEM, Dulbecco's modified Eagle's medium; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperzineethanesulfonic acid; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EDAC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide.

Eagle's medium (DMEM) supplemented with 10% heat-inactivated newborn calf serum. Monolayers of A431 cells were grown to confluency in 15-cm plastic dishes.

Antibodies—Full details concerning the monoclonal antibody 29.1-IgG_1 were described previously (11). The generation and properties of the rabbit antibodies against a synthetic peptide from EGF-R designated RK2-IgG were described elsewhere (12). The rabbit polyclonal anti-phosphotyrosine antibodies were a gift from P. Comoglio (University of Torin).

Receptor Purification—EGF-R was purified from A431 cells essentially as described previously (13), except that all procedures were done in batches of 1.5 ml. Briefly, 2×10^7 A431 cells were solubilized in 1 ml of solubilization buffer (50 mM Hepes buffer, pH 7.5, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM phenylmethanesulfonyl fluoride, 0.15 TIU/ml aprotinin). The cell extract was cleared by centrifugation for 10 min at 4 °C in an Eppendorf centrifuge. The clear extract was mixed with 0.07 ml of monoclonal anti-receptor antibody 29.1-IgG coupled to Sepharose beads (2 mg of antibody/ml of beads). After 1 h at 4 °C the beads were washed three times with 1 ml of HTG buffer (20 mM Hepes, pH 7.5, 0.1% Triton X-100, 10% glycerol), followed by two washes with 1 ml of borate buffer (50 mM borate buffer, pH 8.3, 0.7 M NaCl, 0.3 M $MgCl_2$, 10% glycerol, 0.1% Triton X-100) and two washes with 1 ml of HTG buffer. Radiolabeled receptor was obtained by incubating the receptor bound to the beads for 10 min at 4 °C with [γ-^{32}P]ATP (0.2 μM, 10–50 μCi) and $MnCl_2$ (5 mM) followed by washing with HTG buffer containing phosphatase inhibitors (1 mM ATP, 0.5 mM $ZnSO_4$, and 0.04 mM $NaVO_3$). Elution of the receptor from the immobilized antibody was carried out by either acidic buffer (50 nM glycine-HCl buffer, pH 2.5, 10% glycerol, 0.1% Triton X-100) or by 0.2 M *N*-acetylgalactosamine in HTG buffer. After the acid elution, the purified fraction was neutralized by the addition of concentrated Hepes buffer containing protease inhibitors (1 mM phenylmethanesulfonyl fluoride and aprotinin at 0.15 TIU/ml) and phosphatase inhibitors (0.5 mM $ZnSO_4$ and 0.04 mM $NaVO_3$). Receptor concentration in the purified fraction was determined by binding of radiolabeled EGF followed by polyethyleneglycol precipitation (14) and assuming monovalent interactions between the receptor and EGF. The purified fraction appears mostly as a single M_r 170,000 polypeptide in gels stained by Coomassie Blue.

Cross-linking Experiments in Membranes from A431 Cells—Approximately 1.5×10^8 A431 cells were washed in calcium- and magnesium-free phosphate-buffered saline (PBS) and homogenized at 4 °C in 20 ml of 20 mM Hepes buffer, pH 7.4, containing 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM phenylmethanesulfonyl fluoride, and 10 μg/ml leupeptin. The lysate was centrifuged at $1,500 \times g$ for 10 min, and the supernatant was further centrifuged at $25,000 \times g$ for 30 min. The pellet was resuspended in 20 mM MES buffer, pH 7.0, containing 100 μg/ml leupeptin at a protein concentration of 16 mg/ml. Aliquots of membranes (175 μg of protein) were incubated at 22 °C for 45 min, without or with 200 nM EGF, with gentle agitation. The incubation was continued at 30 °C for 30 min in the absence or presence of 15 mM EDAC (using a freshly made 100 mM solution in MES buffer).

At the end of this incubation, membranes were washed twice with 400 μl of 20 mM Hepes buffer, pH 7.4, containing 100 μg/ml leupeptin and recovered by centrifugation for 10 min at $15,000 \times g$. The membrane pellet was solubilized for 20 min at 22 °C in 10 μl of 20 mM Hepes buffer, pH 7.4, containing 10% glycerol, 100 μg/ml leupeptin, and 0.5% Triton X-100. The extracts were centrifuged for 20 min at $15,000 \times g$, and the resultant supernatants were diluted in 20 μl of 20 mM Hepes buffer, pH 7.4. A 10-μl aliquot of the solubilized receptor was incubated for 10 min at 22 °C with 500 nM EGF and phosphorylated for 10 min at 4 °C with 1 μCi of [γ-^{32}P]ATP (3,000 Ci nmol^{-1}, Amersham International) in the presence of 1 μM cold ATP and 3 mM $MnCl_2$ in a 20-μl final volume. The reaction was stopped by the addition of 5 μl of 5 × Laemmli sample buffer containing 10 mM dithiothreitol. Samples were heated for 3 min at 95% and analyzed on a 3.7% polyacrylamide-SDS gel and autoradiography.

Cross-linking Experiments of Intact A431 Cells—Six plates (28 cm^2) containing approximately 3×10^6 A431 cells were incubated for 30 min at 22 °C with 2 ml of PBS buffer containing 20 mM glucose, 20 μg/ml lactoperoxidase (Boehringer Mannheim), 0.2 units/ml glucose oxydase (Sigma), and 300 μCi of $Na^{125}I$. Cells were washed three times with PBS and incubated for 1 h at 0 °C with 1.5 ml of PBS containing 1 mg/ml bovine serum albumin and 200 nM EGF. Then 10 mM EDAC was added to each well, and the incubation was continued at 30 °C for different times. Cells were then washed rapidly twice with 2 ml of cold PBS and scraped off the plates with 1 ml of RIPA buffer (20 mM Hepes buffer, pH 7.4, 1.5% Triton X-100, 1% deoxycholate, 0.1% SDS, 0.5 M NaCl, 5 mM EDTA, 10 mM EGTA, 1 mM phenylmethanesulfonyl fluoride, 100 μg/ml leupeptin). After 20 min at 0 °C, the extracts were centrifuged for 10 min at $15,000 \times g$. Supernatants were incubated for 2 h at 4 °C with 15 μl of RK II antibody. The solutions were then incubated for 30 min at 22 °C with 20 μl of protein A-Sepharose beads. The immunoprecipitates were washed and the samples were analyzed on a 3.75% polyacrylamide-SDS gel and autoradiography.

Analysis of EGF-R Dimerization by Immunoblotting—Cells were grown to approximately 90% saturation in 24-well dishes in DMEM supplemented with 10% fetal bovine serum and then starved in DMEM containing 25 mM Hepes, pH 7.4, for 16 h. EGF stimulation (100 ng/ml) was conducted in 200 μl of DMEM-Hepes, pH 7.4, for 20 min at 37 °C. In cells treated with cross-linker, 15 mM (EDAC) in 20 mM Hepes, pH 7.8, and 1 μg/ml bovine serum albumin were added for 15 min at 37 °C. Cells were then washed twice with PBS, incubated for 5 min in PBS, and lysed in 100 μl of electrophoresis buffer containing 50 mM Tris-HCl, pH 6.8, 1% SDS, 5% glycerol, and 150 mM glycine. Following protein determination, each was brought to 0.1% basal medium Eagle, heated to 95 °C for 2 min, sonicated for 5 s to decrease viscosity, and subjected to gel electrophoresis in 5% polyacrylamide gels. Proteins were then transferred to nitrocellulose and treated with 1 M glycine for 1 h at 25 °C. The antigen antibody reaction was conducted using previously described procedures, for anti-EGF-R antibody and anti-phosphotyrosine antibodies, and detected by using ^{125}I-protein A and autoradiography (15).

RESULTS

We have shown previously that EGF can induce the formation of receptor oligomers as detected by nondenaturing gel electrophoresis (9).To stabilize the interaction of receptors, in the present study we have used the covalent cross-linker EDAC. This reagent links free amino groups to carboxyl groups which approach each other within the distance of a peptide bond. The products of this cross-linking are covalently linked and, thus, can be analyzed by SDS gel electrophoresis.

We initiated our analysis with attempts to cross-link receptor dimers in systems where receptor oligomerization had previously been detected using native gel electrophoresis. Hence, the EGF-R was incubated for 45 min at 23 °C in the absence or presence of 200 nM of EGF. Half of the samples were then exposed to 15 mM of the covalent cross-linking agent EDAC for 30 min at 30 °C. After washing and solubilization, the membranes were treated with [γ-^{32}P]ATP for internal labeling by receptor autophosphorylation, and the samples were analyzed by SDS-PAGE (Fig. 1*a*). In the presence of EGF and cross-linking agent a phosphoprotein of approximate molecular weight of 340,000 was detected. However, if EDAC is not added a typical M_r 170,000 phosphorylated EGF-R was observed. The 340,000-dalton protein could also be detected by immunoblotting experiments. In these experiments, membranes from A431 cells were incubated with EGF and the cross-linking agent EDAC and then analyzed by SDS-PAGE as before. Subsequently, the samples were transferred to nitrocellulose paper for immunoblotting with anti-EGF-R antibodies. Fig. 1*b* shows that EGF induced the appearance of a M_r 340,000 species which is recognized by the anti-EGF-R antibodies. To determine if the M_r 340,000 polypeptide indeed is composed of EGF-R molecules, the experiment shown in Fig. 1*a* was repeated except that, following the phosphorylation reaction, the samples were reacted with antibodies against EGF-R. Analysis of the immunoprecipitates by SDS-PAGE (Fig. 2) indicated that the M_r 340,000 phosphoprotein is specifically recognized by the EGF-R antibodies. Moreover, the M_r 170,000 and 340,000 proteins have a similar phosphopeptide map (data not shown), indicating that the M_r 340,000 protein contains the EGF-R. Hence, the high molecular weight protein may represent a covalently cross-linked

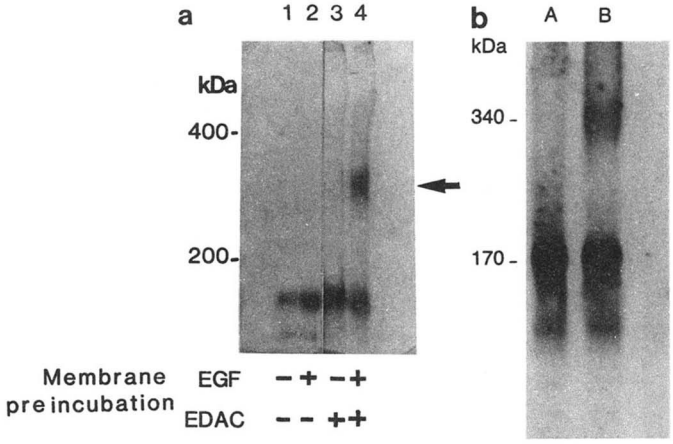

FIG. 1. **EGF-induced receptor dimerization in membranes from A431 cells.** *a*, membranes from A431 cells were incubated with 200 nM EGF (*lanes 2 and 4*) or with buffer alone (*lanes 1* and *3*) for 45 min at 23 °C. The samples were then incubated with the covalent cross-linking agent EDAC (15 mM) (*lanes 3* and *4*) or buffer alone (*lanes 1* and *2*) for 30 min at 30 °C. After washing and solubilization, the samples were subjected to autophosphorylation reaction by incubation with [γ-^{32}P]ATP and $MnCl_2$. The samples were analyzed on 3.7% polyacrylamide-SDS gels and autoradiography. Protein standards included myosin and myosin dimer cross-linked with dimethy suberimidate. *b*, membranes from A431 cells were incubated with 200 nM EGF (*lane B*) or buffer alone (*lane A*) for 45 min at 23 °C and then for an additional 30 min at 30 °C with the covalent cross-linking agent EDAC. After washing, solubilization, and SDS-PAGE, the sample was transferred to nitrocellulose paper and reacted with antibodies against EGF-R and ^{125}I-labeled protein A and analyzed by autoradiography. Arrow in *a* marks the dimeric EGF-R.

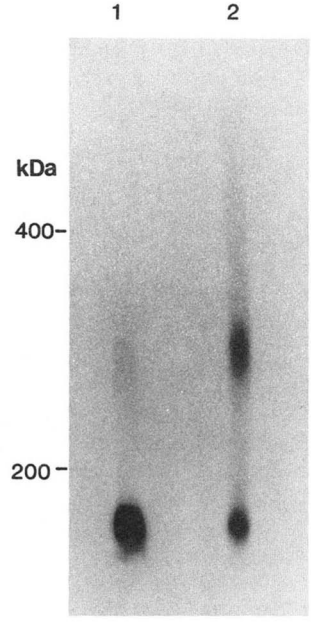

FIG. 2. **Immunoprecipitation of EGF-R monomers and dimers using anti-EGF-R antibodies.** Membranes from A431 cells were incubated with 200 nM EGF (*lane 2*) or with buffer alone (*lane 1*) for 45 min at 23 °C. This was followed by incubation for 30 min with 15 mM EDAC. After washing and solubilization, the samples were subjected to autophosphorylation as described under "Experimental Procedures." The phosphorylated samples were immunoprecipitated with antibodies against EGF-R (RK2) as described previously (12). The samples were analyzed by 3.7% polyacrylamide-SDS gels and autoradiography.

EGF-R dimer or covalently cross-linked heterodimer between EGF-R and an unidentified protein which interacts with the EGF-R. To distinguish between these two possibilities, a similar experiment was performed using purified EGF-R. EGF-R from A431 cells was purified using immunoaffinity chromatography with monoclonal antibodies 29.1 as described previously (13). The 29.1-IgG_1 antibody recognizes carbohydrates which are linked to the EGF-R of A431 cells, and therefore the immobilized receptor was specifically eluted using *N*-acetylgaclatose amine (16). This procedure allows the purification of functional EGF-R with ligand-sensitive protein tyrosine kinase activity (13). The purified EGF-R was internally labeled with ^{32}P and then incubated for 60 min at 4 °C in the absence or presence of 200 ng of EGF. This was followed by an additional 30-min incubation with the covalent cross-linking agent EDAC (15 mM) dissolved in HTG buffer, pH 7.0. The samples were then analyzed on 3.7% polyacrylamide-SDS gels. The results presented in Fig. 3 clearly demonstrate that EGF is able to stimulate the appearance of a protein of M_r 340,000 which is covalently cross-linked with EDAC. Moreover, the M_r 340,000 protein represents an EGF-R dimer captured and preserved by covalent bonds formed by the cross-linking agent.

These results indicate that cross-linking agents can capture the dimer form of EGF-R in isolated systems. Therefore, we were encouraged to determine if EGF-induced dimerization occurs in living cells. We used three approaches to detect such dimers. In the first approach, A431 cells were labeled with [^{35}S]methionine for 16 h and then incubated with 500 ng/ml EGF for 90 min at 4 °C, treated with the cross-linking agent (EDAC), lysed, immunoprecipitated with anti-EGF-R antibodies, and analyzed by SDS-PAGE. Fig. 4 shows that EGF was clearly able to stimulate the appearance of a M_r 340,000 form also in intact A431 cells. Comparison of tryptic peptide maps indicated that the M_r 340,000 form and the M_r 170,000 EGF-R have identical peptide maps (data not shown), indicating that M_r 340,000 proteins represents a dimer of EGF-R. Interestingly, a small but reproducible amount of EGF-R dimers was detected in the absence of EGF (Fig. 4, *lane B*).

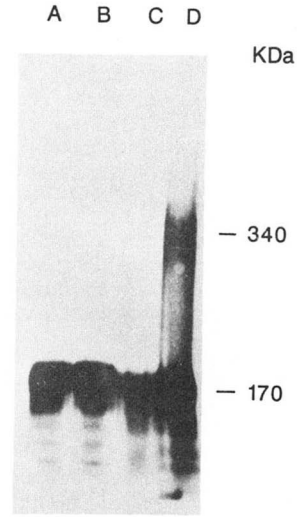

FIG. 3. **EGF-induced dimerization of purified EGF-R.** EGF-R from A431 cells was purified by using immunoaffinity chromatography as described under "Experimental Procedures." Then, the purified EGF-R was internally labeled with ^{32}P and incubated in the presence of 200 ng of EGF (*lanes B* and *D*) or buffer alone (*lanes A* and *C*) for 30 min at 23 °C. This was followed by incubation for 30 min with 15 mM of EDAC (*lanes C* and *D*) or buffer alone (*lanes A* and *B*). The samples were analyzed by 3.7% polyacrylamide-SDS gels and autoradiography.

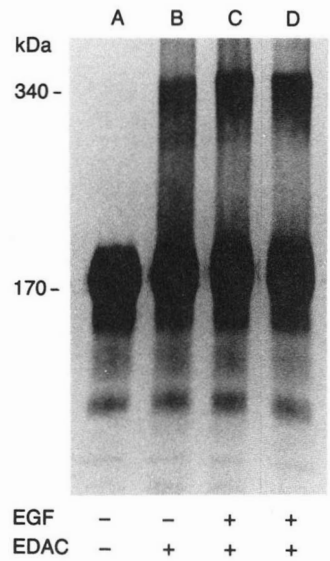

FIG. 4. **EGF-induced receptor dimerization in living biosynthetically labeled A431 cells.** A431 cells were labeled with [^{35}S]methionine for 16 h, incubated with 200 ng/ml (*lane C*) or with 2 μg/ml (*lane D*) EGF for 90 min at 4 °C, treated with EDAC for 30 min at 23 °C, solubilized, immunoprecipitated with anti-EGF-R antibodies, and analyzed by 3.7% polyacrylamide SDS-PAGE and autoradiography.

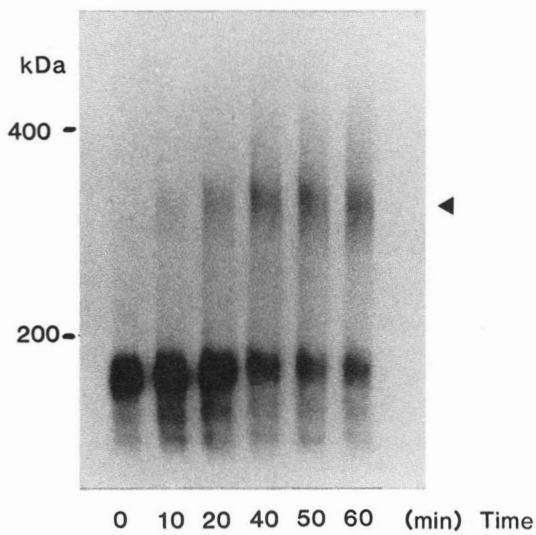

FIG. 5. **EGF-induced receptor dimerization in living surface-iodinated A431 cells.** A431 cells were surface-iodinated as described under "Experimental Procedures." After several washes, the labeled cells were incubated with 200 nM EGF for 1 h at 4 °C. Then, 10 mM EDAC was added, and the samples were incubated at 30 °C for an additional 10–60 min as indicated. After washing and solubilization, the EGF-R was immunoprecipitated with anti-EGF-R antibodies (RK2). The samples were analyzed by 3.7% polyacrylamide-SDS gels and autoradiography. *Arrowhead* marks dimeric EGF-R.

This probably indicates that receptor oligomerization exists also in the absence of EGF.[2] The second approach was to label EGF-R molecules on the surface of A431 cells by using lactoperoxidase and Na^{125}I. Subsequently, the cells were treated as before, and the solubilized samples were immunoprecipitated with anti-EGF-R antibodies and analyzed by SDS-PAGE. Fig. 5 shows that in the absence of EGF a typical M_r 170,000 EGF-R is observed and the EGF was able to stimulate the appearance of a cross-linked product of M_r 340,000. The third approach was to use an immunoblot technique to follow the effect of EGF on receptor dimerization in living cells and to establish the kinetics of receptor dimerization (Fig. 6). In these experiments, living cells were treated with EGF and the cross-linking agent as described previously. After solubilization and SDS-PAGE analysis, the samples were transferred to nitrocellulose paper and then immunoblotted with either anti-EGF-R antibodies or with antibodies against phosphotyrosine. Similar results were obtained when A431 cells or mammary tumor cells (MDA-MB468) were analyzed by this method (data not shown). Since the EGF-R undergoes rapid self-phosphorylation, the anti-phosphotyrosine antibodies will allow the comparison of the phosphotyrosine content of the dimeric and monomeric receptors. Fig. 6 shows that, in the presence of EGF, both antibodies recognize the M_r 170,000 EGF-R and an EGF-induced M_r 340,000 dimeric receptor and that dimerization is a rapid process as receptor dimers are clearly visible after 5 min incubation with EGF (Fig. 6*c*). Hence, using three independent approaches it is possible to conclude that EGF is able to rapidly stimulate the appearance of a M_r 340,000 polypeptide in living A431 cells. This polypeptide is phosphorylated on tyrosine residues and probably represents a dimeric EGF-R similar to the dimeric receptor detected in experiments performed with either solubilized or purified EGF-R.

DISCUSSION

According to the allosteric aggregation model (3, 8, 9), the binding of EGF to its receptor stabilizes a receptor-oligomeric state which possesses elevated protein tyrosine kinase activity. Receptor dimers were detected when purified EGF-R was analyzed in nondenaturing gels (9). Moreover, receptor oligomerization was detected in membrane preparations (18) and in intact cells (17, 19). The life time of EGF-R dimers is long enough to allow quantitative separation of receptor dimers and monomers using native gels. Hence, using this approach it was shown that receptor dimerization induced by EGF is a rapid, reversible, saturable and temperature sensitive process (9). In the current study we have extended this observation of EGF-induced dimers to living cells. We show that when EGF is applied to A431 cells, dimers of receptor are formed from cell surface receptor in a rapid fashion. Our strategy was to capture and preserve the receptor dimers formed in response to EGF using the chemical cross-linker EDAC. EDAC is known to bridge lysine side chains and acidic residues which are within distance of a peptide bond. We first analyzed the effectiveness of this cross-linker to substantiate earlier results using native gel electrophoresis. We demonstrate that in membrane preparations EDAC traps a M_r 340,000 form of the receptor when EGF is present. This form is immunoreactive with anti-EGF-R antibodies both in immunoprecipitation and immunoblotting methods. A M_r 340,000 form is also trapped by cross-linked preparations of purified EGF-R. This strongly suggests that the M_r 340,000 form is indeed a dimeric receptor.

Three independent approaches were utilized to follow EGF-R dimerization in living cells. These included biosynthetic labeling, surface iodination, and immunoblotting experiments. Only the surface iodination experiments provide some insight into the relative amounts of receptor dimers and monomers which occur in response to EGF. The two other techniques underestimate the proportion of EGF-R dimers since both methods label the entire pool of EGF-R, including the intracellular EGF-R which is inaccessible to EGF. In the

[2] I. Lax, F. Bellot, A. M. Honegger, A. Schmidt, A. Ullrich, D. Givol, and J. Schlessinger (1987) submitted for publication.

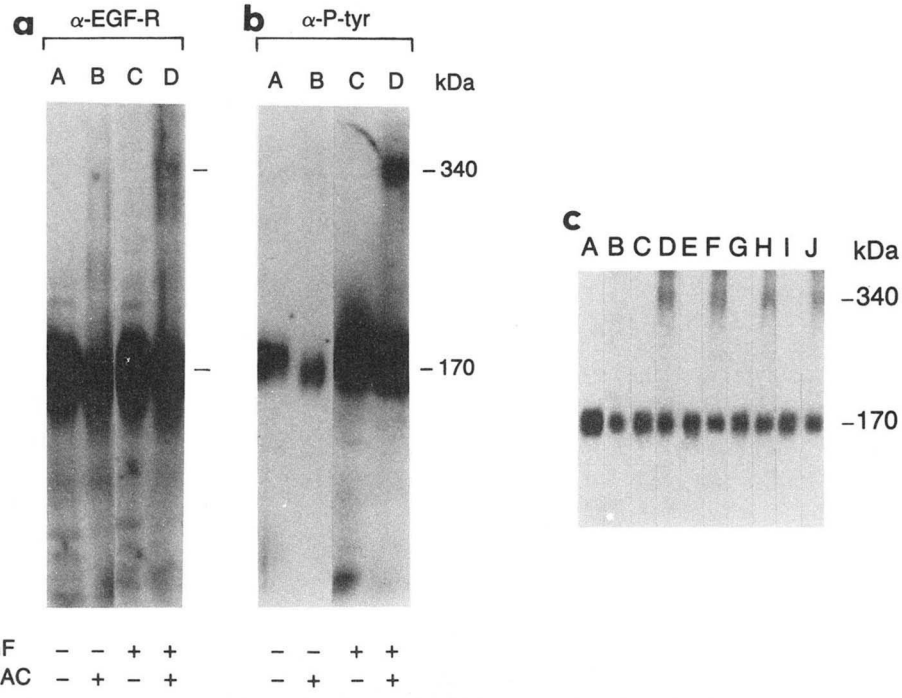

FIG. 6. **EGF-induced receptor dimerization in living cells determined by immunoblotting experiments.** Cells were incubated with 500 ng/ml EGF for various times as indicated and then treated with 15 mM EDAC for 15 min at 37 °C and solubilized. Following SDS-PAGE and transfer to nitrocellulose paper, the samples were blotted by anti-EGF-R or antiphosphotyrosine antibodies and ^{125}I-labeled protein A and analyzed by autoradiography. *a*, A-431 cells incubated with EGF for 5 min at 37 °C, and samples were blotted with anti-EGF-R antibodies (RK2). *b*, A-431 cells incubated with EGF for 5 minutes at 37 °C and samples were blotted with antiphosphotyrosine antibodies (*α-P-tyr*). *c*, kinetics of dimerization in living cells. A-431 cells were incubated with 500 ng/ml EGF at 37 °C for increasing periods of time and then treated with EDAC and analyzed as before by blotting with anti-EGF-R antibodies (RK2). *Lanes A* and *B*, no EGF; *lanes C* and *D*, 5 min incubation; *lanes E* and *F*, 10 min; *lanes G* and *H*, 30 min; and *lanes I* and *J*, 60 min. *Lanes A, C, E, G*, and *I* are in the absence of EDAC, and *lanes B, D, F, H*, and *J* are in the presence of EDAC.

immunoblotting experiments it is also assumed that the monomeric and dimeric EGF-R are equally well transferred to the nitrocellulose paper and that both species are equally well recognized by the anti-EGF-R antibodies. Moreover, the yield of the covalent cross-linking reaction is probably low, and therefore, it cannot be applied for quantitation of receptor dimerization. As a whole, these approaches should be considered as qualitative rather than quantitative methods for the determination of EGF-R dimerization on living cells, and as such, they clearly provide a consistent picture; namely, that EGF stimulates rapidly the appearance of EGF-R dimers in living cells. We have shown that it is possible to detect a small number of receptor dimers also in the absence of EGF (Fig. 4). This is consistent with recent results from our laboratory in which we have demonstrated EGF-R oligomerization in the absence of EGF.[2] This study is based on immunoprecipitation experiments with site- and species-specific antibodies revealing an association between mutant and native EGF-Rs when both proteins are expressed in the same cell.[2] Hence, it appears that receptor oligomerization occurs in the absence of ligand binding and that the binding of EGF stabilizes the oligomeric state.

What is the functional role of EGF-induced receptor dimerization? The allosteric aggregation model (3, 8, 9) predicts that receptor dimers have elevated protein tyrosine kinase activity. Unfortunately, we do not have yet an answer to this question. Yarden and Schlessinger (9) obtained some evidence, using native gel systems to separate between receptor dimers and monomers, that the dimeric EGF-R is phosphorylated earlier than the monomeric EGF-R. However, because of the reversible nature of receptor oligomerization, it was impossible to quantitate the phosphorylation state of the monomeric and dimeric EGF-R separately. Our results demonstrate that both receptor dimers and monomers are phosphorylated on tyrosine residues. Experiments presented in Fig. 6 may provide a potential tool for addressing the question of the relative kinase activity of the monomeric and dimeric forms of EGF-R. In this experiment, the monomeric and dimeric receptors induced by EGF were analyzed by an immunoblot technique applying antibodies either against EGF-R or against phosphotyrosine. Both antibodies recognized the monomeric and dimeric EGF-R separately. Quantitation of the experiments shown in Fig. 6 indicated that the cross-linked dimeric receptor is slightly more phosphorylated than the monomeric receptors, suggesting that the dimeric state of the receptor has elevated kinase activity. However, the use of this technique is limited by the low efficiency of the cross-linking reaction. Low efficiency cross-linking would tend to underestimate the amount of dimeric receptor. Moreover, the anti-EGF-R antibodies interact with the entire pool of EGF-R including the intracellular receptors which cannot interact with EGF, while the phosphotyrosine antibodies recognize only the phosphotyrosine labeled receptors; namely, the activated EGF-R molecules. Hence, using this technique we cannot at present adequately compare the relative phosphorylation of the monomeric and dimeric receptors in living cells. Nevertheless, improvement in the yield of the cross-linking reaction combined with this method for studying purified

EGF-R may provide a potential approach for addressing this question in future studies.

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