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Domain deletion in the extracellular portion of the EGF-receptor reduces ligand binding and impairs cell surface expression

I. Lax,* F. Bellot,* A.M. Honegger,* A. Schmidt,* A. Ullrich,† D. Givol,§ and J. Schlessinger*§
*Rorer Biotechnology, Inc., King of Prussia, Pennsylvania
†Max Planck Institut für Biochemie, 8033 Martinsried bei München, Federal Republic of Germany
§Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel

Cultured NIH-3T3 cells were transfected with cDNA constructs encoding human epidermal growth factor–receptor (EGF-R)* and two deletion mutants in the extracellular portion of the receptor molecule. One mutant is devoid of 124 amino-terminal amino acids, and the other lacks 76 residues. Mutant receptors were not delivered to the cell surface unless the transfected cells contained also endogenous EGF-Rs, suggesting that receptor interaction complements the mutation and allows surface display of mutant receptors. Immunoprecipitation experiments revealed an association between mutant and endogenous EGF-Rs when both proteins were expressed in the same cell. Hence, receptor-oligomers may exist in the plane of the membrane even in the absence of ligand binding, and oligomerization may play a role in normal trafficking of EGF-Rs to the cell surface.

Mutant receptors retained partial ligand binding activity as ^{125}I-labeled EGF was covalently cross-linked to both mutant receptors, and EGF stimulated, albeit weakly, their protein tyrosine kinase activity. Both mutant EGF-Rs bind EGF with a 10-fold lower affinity than that of the solubilized wild type EGF-R. These results provide further evidence that the region flanked by the two cysteine-rich domains plays a crucial role in defining ligand-binding specificity of EGF-R.

* Abbreviations: DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; HEPES, 4-(hydroxyethyl)-piperazine-ethanesulfonic acid; HNTG, HEPES, NaCl, glycerol, and Triton X-100; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecylsulfate.

Introduction

The first step in the action of the mitogenic polypeptide epidermal growth factor (EGF) is specific binding to a surface glycoprotein known as the EGF-receptor (EGF-R) (reviewed in Carpenter and Cohen, 1979; Schlessinger *et al.*, 1983, Schlessinger 1986; Yarden and Ullrich, 1988). The mature EGF-R is composed of 1186 amino acid residues that are preceded at the N-terminal end by a signal peptide (Ullrich *et al.*, 1984). The signal peptide is cleaved after the insertion of the nascent receptor into the membrane of the endoplasmic reticulum. The EGF-R is cotranslationally glycosylated and transported via the Golgi apparatus to the plasma membrane. Binding of EGF to the extracellular receptor domain results in activation of the cytoplasmic protein tyrosine kinase domain (Ushiro and Cohen, 1980), which undergoes autophosphorylation and phosphorylates cellular protein substrates, which are thought to be part of the EGF-R signal transduction pathway (reviewed in Hunter and Cooper, 1985). Indeed, it was shown that the kinase activity of EGF-R is essential for signal transduction, mitogenesis, transformation and normal cellular routing (Livneh *et al.*, 1986b, 1987; Honegger *et al.*, 1987a,b; Chen *et al.*, 1987). The extracellular ligand binding portion is composed of 621 amino acid residues. It contains numerous N-linked glycosylation sites and two cysteine-rich domains that can be aligned as internal repeats with similar spacing between individual cysteine residues. Two additional domains can be defined in the extracellular portion of EGF-R; one N-terminal to the first cysteine-rich domain and a second one between the two cysteine-rich domains, each consisting of ~150 amino acids (Lax *et al.*, 1988b). Using affinity-labeled experiments and mutational analysis, we have demonstrated that the region flanked by the two cysteine domains is a major ligand binding region of EGF-R (Lax *et al.*, 1988a, 1989).

As part of our efforts to define the function of various receptor domains and specifically to characterize the EGF-R ligand binding site, we

describe here the generation and properties of two deletion mutants in the first domain of the extracellular portion of the human EGF-R. The mutant receptors retained partial ligand-binding capacity, as ^{125}I-labeled-EGF can be covalently cross-linked to mutant receptors, and EGF was able to stimulate the protein tyrosine kinase activity of the mutant receptors. Immunoprecipitation experiments with various anti-EGF-R antibodies revealed an association between the human EGF-R deletion mutants and the native murine EGF-R. The association between mutant and native EGF-R appears to be essential for the display of the mutant receptors on the cell surface, because, in cells devoid of native EGF-R, the deletion mutants remain in intracellular compartments and are not transported to the cell surface.

Results

Expression of deletion mutants in NIH-3T3 cells

We have generated two deletion mutants in the extracellular ligand-binding domain of the human EGF-R following the procedure outlined in Figure 1. Deletions were introduced downstream from the signal sequence to yield constructs missing 228 nucleotides coding for amino acids 1–76 (pHERD76N) or 372 nucleotides coding for amino acids 1–124 (pHERD-124N). These deletions leave the signal peptide and its cleavage site intact, and residues 77 and 125 are the N-terminal residues of the two constructs, respectively. Mutated cDNAs were cloned into the modified expression plasmid pLSV under the control of the SV40 early promoter (Livneh *et al.,* 1986b). These plasmids were cotransfected into NIH-3T3 cells with a plasmid containing the neomycin resistance gene (pSVNeo), and this was followed by Geneticin (G-418) selection. Resistant cells were then analyzed for the expression of the receptor mutants. The NIH-3T3 cells used for transfection were previously characterized and shown to express undetectable amounts of endogenous murine EGF-Rs (Livneh *et al.,* 1986b; 1987; Honegger *et al.,* 1987a,b). Several cloned cell lines expressing wild type receptor or deletion mutants were developed. All experiments presented in this study were repeated with at least two cell lines expressing the various constructs, with essentially the same results. Four different antibodies against EGF-R were used for the characterization of the EGF-R and its mutant. These include two monoclonal antibodies specific for the human EGF-R; mAb108, which blocks the binding of EGF only to the high-affinity binding sites; and mAb-96, which blocks EGF binding to EGF-R (Bellot *et al.,* submitted). Two different rabbit antipeptide antisera were also used. One (RK2) is an antiserum directed against a synthetic peptide from the cytoplasmic domain of the EGF-R that recognizes both murine and human EGF-R (Kris *et al.,* 1985). The second one is an antiserum raised against a synthetic peptide representing the N-terminal peptide (residues 1–18, Ab-N) of the mature human EGF-R (Ullrich *et al.,* 1984). This antibody recognizes human EGF-R but not murine EGF-R.

Figure 2 depicts sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of [^{35}S]methionine-labeled cells subjected to immunoprecipitation with the antipeptide antiserum RK2. It shows that the constructs pHERD76N and pHERA124N direct the synthesis of polypeptides of M.W. 150 000 and 135 000, respectively (Figure 2, lanes a and b). We have also generated cell lines that express wild type human EGF-R (Figure 2, lane d) and cell lines that express deletion mutant pHERD-124N and also contain native murine EGF-R (Figure 2, lane e). In this cell line, both murine EGF-R and the human mutant were immunoprecipitated by antibody RK-2. Figure 2 also shows that the level of expression of wild type and mutants EGF-R is similar in these cell lines and that parental untransfected NIH-3T3 cells contain undetectable amounts of EGF-R.

To determine the amount of carbohydrate linked to the EGF-R mutants, the transfected cell lines were labeled with [^{35}S]methionine, treated with either monensin or tunicamycin, and then subjected to immunoprecipitation with RK2 antiserum and analyzed by SDS-PAGE (Figure 3). In the presence of monensin or tunicamycin, the apparent M.W. of the wild type EGF-R decreased from 170 000 to 155 000 and 135 000, respectively. Hence, the apparent M.W. of the protein core of the human EGF-R expressed in NIH-3T3 cells (135 000) is identical to the M.W. of the protein core of native human EGF-R expressed in either A-431 cells or in human foreskin fibroblasts (Stoscheck and Carpenter, 1984). Using this approach, we have determined the M.W. of the protein core of deletion mutant pHERD76N to be 115 000 and of deletion mutant pHERD124N to be 110 000 (Figure 3). The deletion in pHERD76N did not contain any potential glycosylation site, and the tunicamycin treatment decreased its apparent M.W. by 35 000, similar to the decrease in the wild type receptor. The deletion in pHERD124N

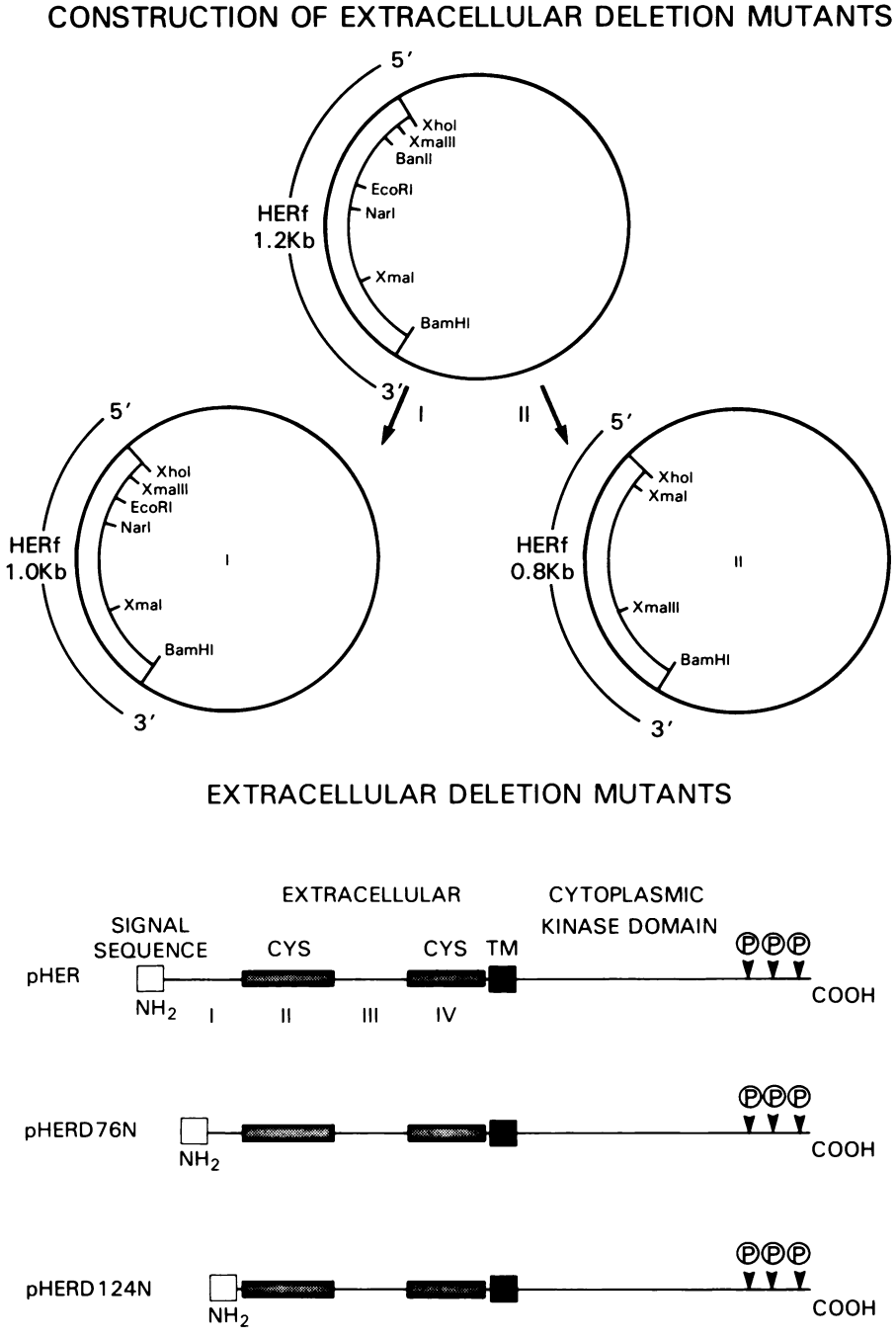

***Figure 1.* Structure of EGF-R and two deletion mutants.** (A) Steps in the construction of extracellular deletion mutants. For details see Materials and Methods section. (B) Structure of wild type human EGF-R construct (pHER) and two deletion mutants, pHERD76N and pHERD124N. The signal sequence, two cysteine-rich domains, the transmembrane region, the kinase domain, and autophosphorylation sites are indicated. The extracellular portion is divided to four domains as indicated: Domain I, the region from the amino-terminal residue up to the first cysteine-rich region; Domain II, the first cysteine-rich region; Domain III, the region flanked by the two cysteine-rich domains; and Domain IV, the second cysteine-rich region.

contains one potential glycosylation site, and tunicamycin treatment decreased its apparent M.W. by 25 000 daltons.

Murine EGF-R is co-immunoprecipitated with EGF-R deletion mutants

Monoclonal antibodies mAb108 and mAb-96 recognize the human EGF-R and its deletion mutants but not the murine EGF-R (Figure 4). Nevertheless, with the use of these antibodies it is possible to immunoprecipitate the murine EGF-R from solubilized cells, which possess pHERD124N mutant receptors and murine EGF-R (Figure 4I, lane D), as efficiently as with anti-peptide antibodies RK2, which recognize equally well both human and murine EGF-R (Figure 2, lane 3). Similar results were obtained when these antibodies were used for the immunoprecipitation of phosphorylated EGF-R (data not

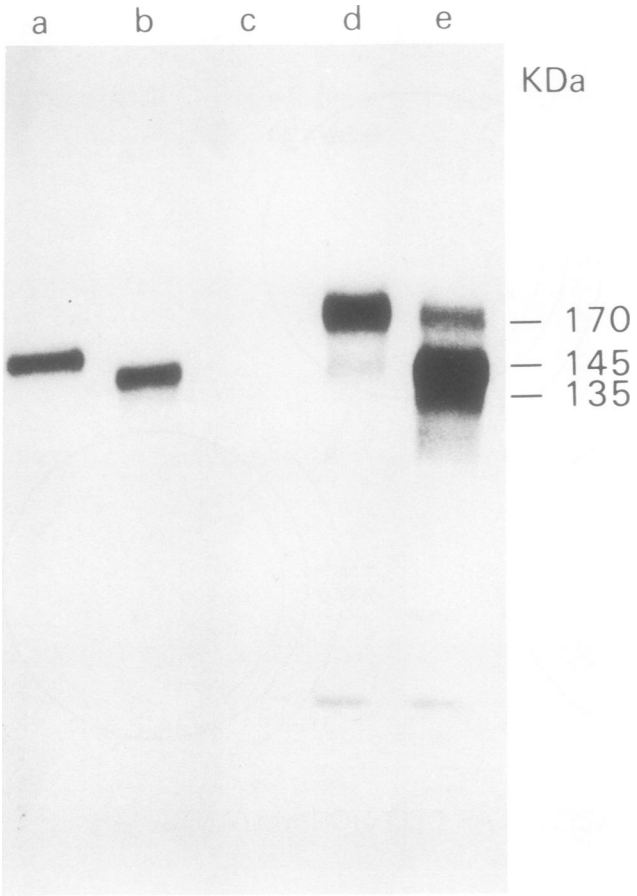

Figure 2. **Identification of wild type EGF-R and deletion mutants by immunoprecipitation of [^{35}S]methionine labeled cells.** Cells were labeled overnight with [^{35}S]methionine, lysed, and immunoprecipitated with RK2 anti-EGF-R antibodies as described in the Methods section. The samples were analyzed by SDS-PAGE with the use of 6.5% gel and autoradiography. Lane a, NIH-3T3 cells expressing deletion mutant pHERD76N; lane b, NIH-3T3 cells expressing deletion mutant pHERD124N; lane c, parental NIH-3T3 cells; and lane d, NIH-3T3 cells expressing wild type EGF-R; and lane e. NIH-3T3 cells expressing both murine native EGF-R and deletion mutant pHERD-124N.

shown). Hence, these experiments show that murine EGF-R is co-immunoprecipitated with the human EGF-R deletion mutants. We interpret these results as an indication that EGF-R molecules are held together in the plane of the membrane and that this association is maintained also between EGF-R of different species when coexpressed in the same cell. To further examine this hypothesis, both EGF-R deletion mutants were transiently expressed in COS-7 cells, which contain endogenous monkey EGF-R (Livneh *et al.,* 1986a). Transfected cells were lysed and subjected to immunoprecipitation experiments with two different site-specific antipeptide antisera. The first antibody is directed against a synthetic peptide corresponding to the N-terminal sequence of EGF-R (Ab-N), which recognizes the monkey EGF-R and not the human mutants because of the deletion of their N-terminus. The second antipeptide antiserum RK2 recognizes both the monkey and the human EGF-R deletion mutants. Figure 4II shows that the anti-N-terminal antibodies are as efficient as RK2 antibodies in immunoprecipitating the human EGF-R deletion mutants. This result is consistent with the hypothesis that EGF-R molecules are held together in the plane of the membrane and that this association is responsible for the co-immunoprecipitation of native and mutant EGF-Rs.

Deletion mutants are not expressed on the cell surface unless associated with native EGF-R

To determine the levels of EGF-R on the surface of intact cells, we have used murine ^{125}I-labeled EGF, which binds equally well to human and murine EGF-R, and monoclonal antibody mAb108, which binds to human but not to murine EGF-R. Figure 5 shows that HER1 cells expressing wild type human EGF-R display on their surface 6×10^5 EGF-R molecules and that the human EGF-R is also recognized by mAb108. Similar binding experiments utilizing mAb108 indicated that both deletion mutants are not expressed on the cell surface of the transfected NIH-3T3 cells (Fig. 5). Surprisingly, surface localization of deletion mutant pHERD124N is observed when this mutant is expressed in

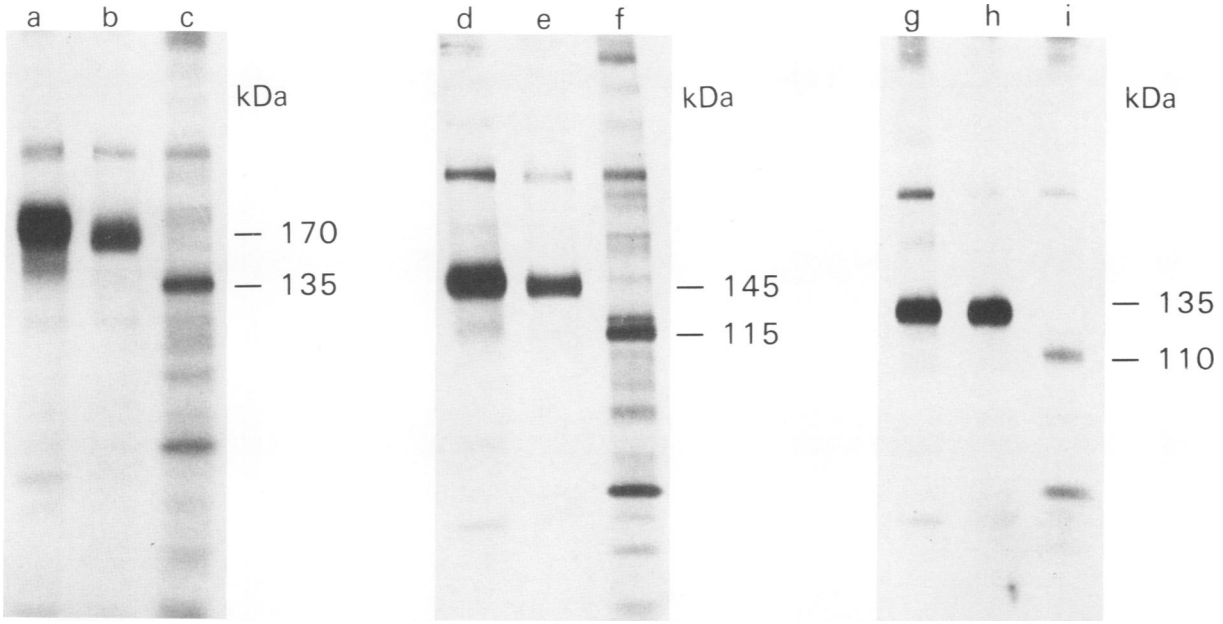

Figure 3. **Effect of monensin and tunicamycin on wild type or EGF-R mutants.** Cells were treated overnight with [^{35}S]methionine (lanes a, d, and g are controls), in the presence of 30 μg/ml monensin (lanes b, e, and h) or in the presence of 10 μg/ml tunicamycin (lanes c, f, and i), then lysed and immunoprecipitated with RK-2 anti-EGF-R antibodies as described in the Methods section. The samples were analyzed by SDS-PAGE with the use of 6.5% gel and by autoradiography. (I) NIH-3T3 cells expressing wild type receptor (HER1 cells); (II) NIH-3T3 cells expressing deletion mutant (NpHERD76N cells); and (III) NIH-3T3 cells expressing the second deletion mutant (NpHERD124N cells).

NpHERD124NI cells, which possess endogenous murine receptors (Figure 5). These cells contain on their surface 35 000 ± 5000 (SD) murine EGF-R and 100 000 ± 35 000 (SD) molecules of deletion mutant pHERD124N, as measured by antibody binding.

The inability of the deletion mutants to be transported to the cell surface was also revealed by studying the cellular localization of the deletion mutants by the use of immunofluorescence microscopy (Figure 6). The EGF-R deletion mutant pHERD124N cannot be visualized unless the cells are first permeabilized with 0.1% Triton X-100. When the permeabilized cells are fluorescently labeled with mAb108, they show typical intracellular staining, indicating cytoplasmic localization of the EGF-R deletion mutant, probably in the endoplasmic reticulum (Figure 6, a–d). When a similar experiment was performed with cells that also express endogenous murine EGF-R, a different pattern was observed. Fluorescence staining with mAb108, which recognizes only human EGF-R, revealed typical surface labeling of fluorescent antibody, indicating that the EGF-R deletion mutants are expressed on the cell surface of these cells. An additional conclusion that can be drawn from these experiments is that, although present on the cell surface, the binding affinity of the deletion mutant toward EGF is too low to be determined by conventional ^{125}I-EGF binding experiments. We therefore analyzed the binding affinity of EGF to the mutant EGF-R in solubilized cells by the use of a different approach.

Once coexpressed on the cell surface together with native EGF-R, the amount of the mutant receptor on the cell surface can be regulated by EGF (Carpenter and Cohen, 1976; Schlessinger *et al.*, 1978; Haigler *et al.*, 1978). Figure 7A shows that the addition of EGF to these cells caused down regulation of both mutant and native EGF-R, with kinetics similar to the down regulation and degradation of wild type EGF-R induced by ligand binding (Carpenter and Cohen, 1976; Honegger *et al.*, 1987a). We have also analyzed the capacity of EGF to stimulate receptor degradation. Transfected cells were labeled with [^{35}S]methionine for 15 h, then incubated with EGF for increasing periods of time, and subsequently lysed and subjected to immunoprecipitation and SDS-PAGE analysis. Both wild type and mutant EGF-R have similar half-life of 10–12 h (Honegger *et al.*, 1987a). The addition of EGF to cells expressing wild type EGF-R decreased its half-life to ~1 h (Honegger *et al.*, 1987a). A similar result was obtained when EGF

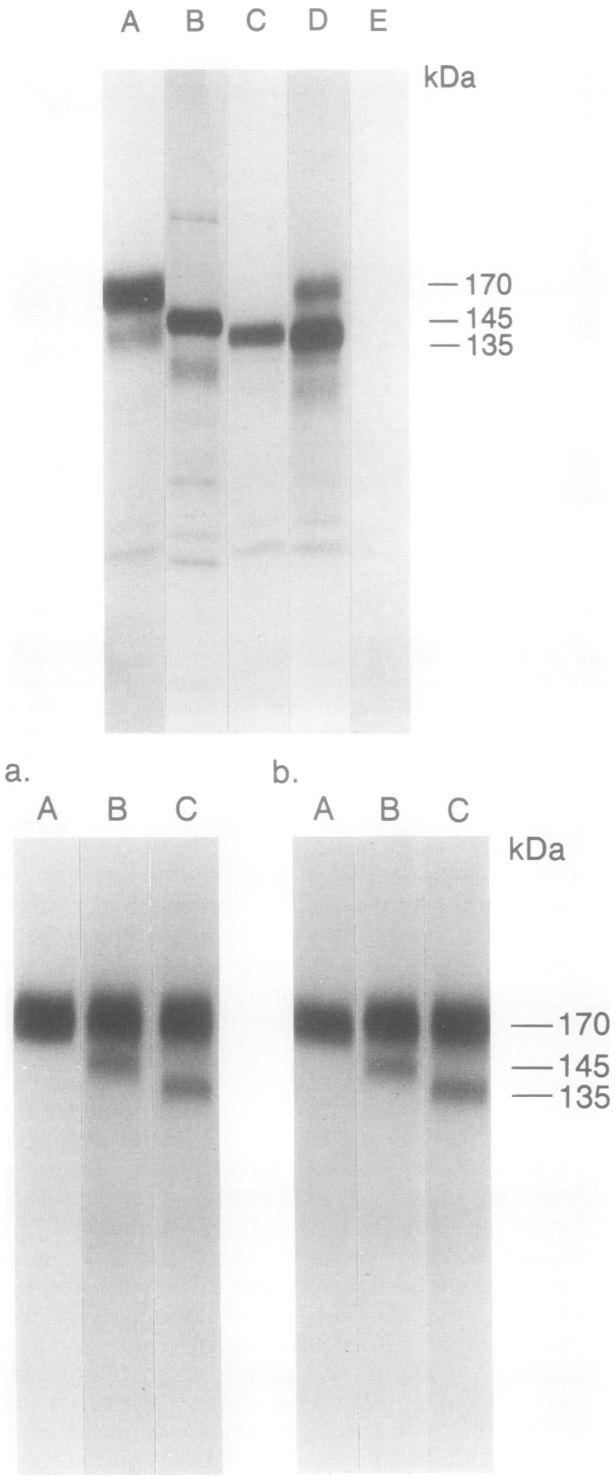

Figure 4. **Co-immunoprecipitation of wild type and mutant EGF-R.** (I) Cells labeled overnight with [^{35}S]methionine were lysed and immunoprecipitated with mAB108 (recognizes human but not mouse EGF-R) as described in the Methods section. Lane A, NIH-3T3 cells expressing wild type receptors (HER1 cells); lane B, NIH-3T3 cells expressing deletion mutant (NpHERD76N cells); lane C, NIH-3T3 cells expressing both endogenous murine EGF-R and deletion mutant (NpHERD124NI cells); and lane E, parental NIH-3T3 cells. The weak bands beneath the major polypeptide bands are degradation products of EGF-R, and of its deletion mutants. (II) Constructs encoding EGF-R deletion mutants were transiently expressed in COS-7 cells, which contain endogenous monkey EGF-R. The transfected cells were lysed and subjected to immunoprecipitation experiments with two site-specific antipeptide antisera as described in the Methods section. (a) RK-2 antiserum, which recognizes both monkey and human EGF-R, (b) Ab-N antiserum, which recognizes only the monkey EGF-R and not the two deletion mutants of human EGF-R. Lane A, mock transfected COS-7 cells; lane B, COS-7 cells transfected with construct pHERD76N; and lane C, COS-7 cells transfected with construct pHERD124N. All samples were analyzed by SDS-PAGE with the use of 6.5% gel and by autoradiography.

was added to cells expressing both native murine and mutant EGF-R (Figure 7B). In these cells, in the presence of EGF, the mutant receptors are degraded with similar kinetics to that of native or wild type receptors with a half-life of ~1 h.

EGF-R deletion mutants retain capacity for covalent cross-linking of ^{125}I-EGF

To examine the ability of EGF-R deletion mutants to interact with EGF, we have used covalent cross-linking experiments with ^{125}I-EGF.

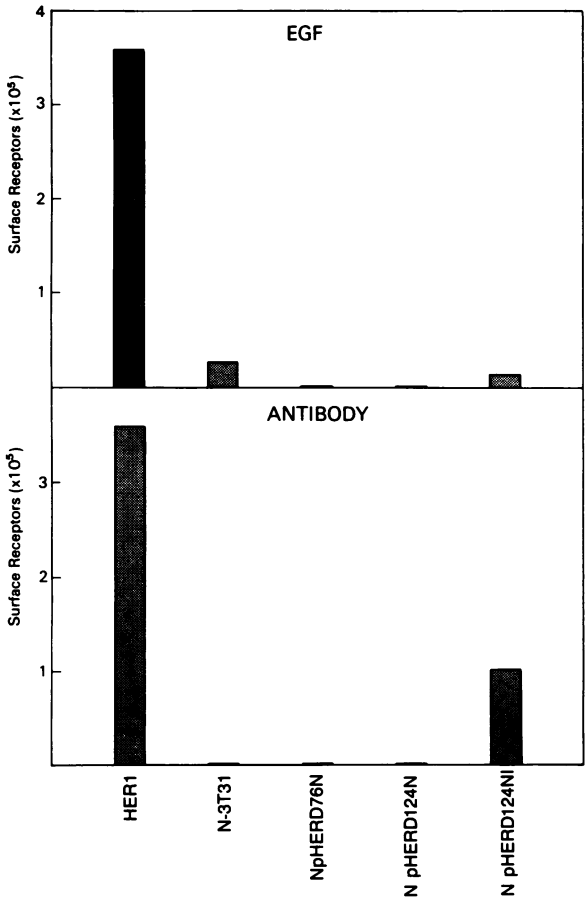

Figure 5. **Quantitation of EGF-R expressed on the surface of various cells with the use of ^{125}I-EGF (binds to human and mouse EGF-R) and ^{125}I-labeled mAb108 (binds to human but not to murine EGF-R) as specific probes.** Top panel, EGF binding data; bottom panel, mAb108 binding data. HER1 cell, NIH-3T3 cells devoid of endogenous murine EGF-R expressing wild type human EGF-R; N-3T31 cells, NIH-3T3 cells expressing endogenous murine EGF-R expressing deletion construct pHERD76N; NpHERD124N cells, NIH-3T3 cells devoid of endogenous murine EGF-R expressing deletion construct pHERD124N; NpHERD124NI, NIH-3T3 cells expressing endogenous murine EGF-R and deletion construct pHERD124N.

According to this method (Linsley and Fox, 1980), incubation of chloramine-T–radiolabeled ^{125}I-EGF results in covalent cross-linking of ^{125}I-EGF to EGF-R. This approach provides a useful and reliable means for labeling the extracellular domain of the EGF-R. Figure 8 shows that immunoprecipitated EGF-R deletion mutants retain the capacity for covalent cross-linking of ^{125}I-EGF. This reaction is specific and can be blocked by the addition of excess unlabeled EGF (Figure 8II). Moreover, immunoprecipitates from parental NIH-3T3 cells and related proteins such as the v-erbB oncogene product (truncated avian EGF-R) and HER2/*neu* proto-oncogene product (an EGF-R–related protein) were not covalently conjugated with ^{125}I-EGF (Figure 8I).

We could not determine the ligand-binding affinity of the mutant EGF-R with the use of conventional ^{125}I-EGF binding experiments for the following reasons. First, the mutant receptors are not expressed on the cell surface; second, binding experiments with ^{125}I-EGF to solubilized preparation suffer from high nonspecific binding; and third, conventional binding experiments allow the determination of only a limited range of dissociation constants.† Therefore, we have determined the ligand-binding affinities of the deletion mutants, and as a control of wild type EGF-R, by measuring the ID_{50} (concentration of ligand at 50% inhibition) for the inhibition of covalent cross-linking of ^{125}I-EGF by unlabeled EGF. Figure 8II shows the effect of increasing concentration of unlabeled EGF on the covalent cross-linking of ^{125}I-EGF to wild type and mutant EGF-Rs. The ligand-binding affinity of wild type EGF-R as determined by this assay is $K_d = 1.3 \times 10^{-8}$ M, a value that is in good accord with the dissociation constant of the solubilized EGF-Rs determined by Scatchard analysis of ^{125}I-EGF binding data (Yarden and Schlessinger, 1985). The dissociation constant of both deletion EGF-R mutants was found to be $(1–2) \times 10^{-7}$ M. Hence, deletion of domain I decreases the binding affinity for EGF by ~10-fold.

Stimulation of kinase activity of EGF-R deletion mutants by EGF

The kinase activity of wild type EGF-R and its deletion mutants was analyzed. Lysates prepared from the various cell lines were incubated

† The association constant of ligand to receptor molecule, $Ka = k_{on}/k_{off}$, where k_{on} and k_{off} represent the forward and backwards rate constants for ligand binding, respectively. The k_{off} of EGF for EGF-R is estimated to be $\sim 10^{-3}$ s^{-1}, and therefore, $k_{on} = K_a \times k_{off} = 10^{10} \times 10^{-3} = 10^7$ M^{-1} sec^{-1}. This value is 100-fold smaller than the theoretical diffusion control value for $k_{on} \sim (10^9$ M^{-1} sec$^{-1})$, a difference that is due to the probability of trajectory finding the receptor sites, which needs to be taken in account (Tanford, 1961, Sharp *et al.*, 1987). Hence, if we assume that k_{on} of deletion mutant and wild type receptors are similar because both are essentially diffusion controlled (Weber, 1975; Koren and Hammes, 1976), then the lower binding affinity of mutant receptor is mainly due to a decrease in its k_{off}. Hence, a 1000-fold decrease in k_{off}, which corresponds to $k_d = 10^{-7}$ M^{-1}, will mean that half-life time of dissociation $T = 1/k_{off} < 1$ second. Hence, bound ligand will dissociate from the receptor during the washes, which are necessary for the release of nonspecific radioactivity. This will set an upper limit for the determination of ligand binding constant when utilizing this approach. Therefore, the upper limit for measurement of the K_d of EGF binding to mutant receptor is $K_d \leq 10^{-7}$ M.

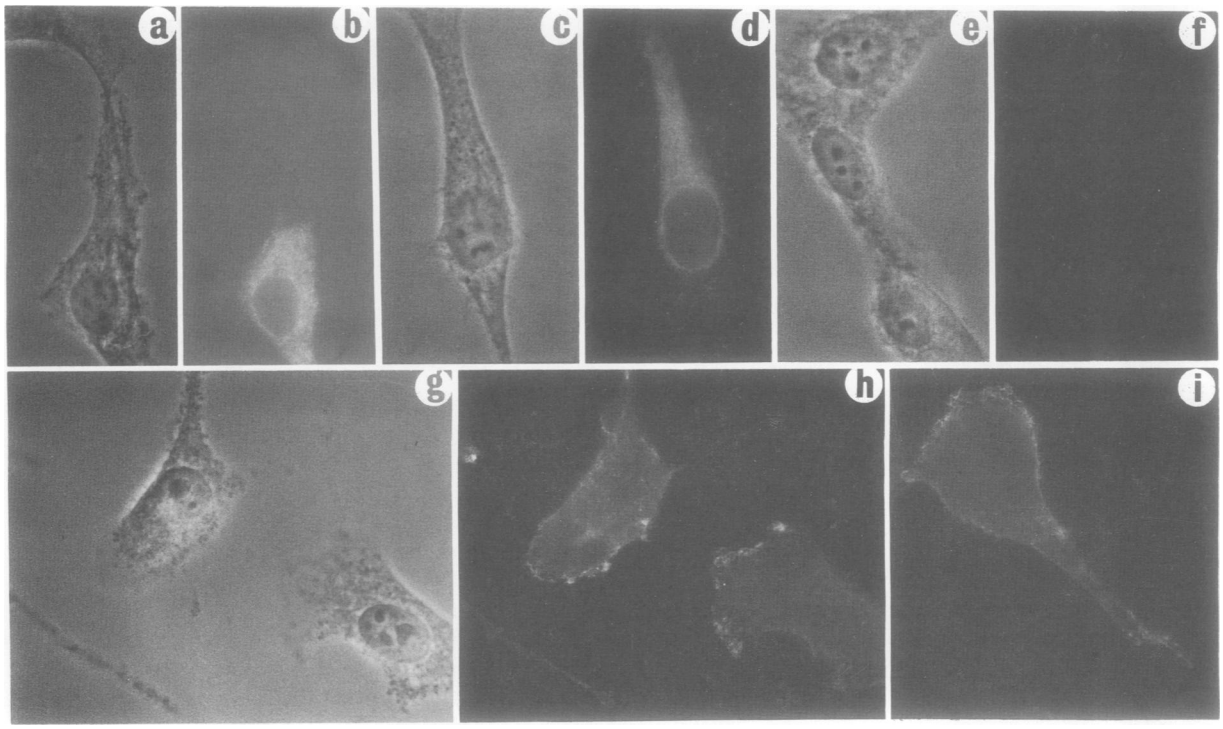

Figure 6. **Fluorescence micrographs of transfected cells labeled with mAb108 and fluorescein labeled rabbit anti-murine Ig antibodies.** Transfected cells were labeled with mAb108 (binds to human but not to murine EGF-R) and then with fluorescein conjugated rabbit anti-mouse Ig antibodies. Panels a–d: NIH-3T3 cells expressing deletion mutant pHERD124N. These cells had to be permeabilized with 0.1% Triton X-100 for receptor visualization using the fluorescent antibodies. Panels a and c are phase and panels b and d are fluorescence micrographs of the same fields, respectively. Panels e and f: Permeabilized and fluorescently immunolabeled parental untransfected NIH-3T3 cells. Panels g and h: NIH-3T3 cells that express both native murine EGF-R and deletion mutant pHERD124N. These cells were not permeabilized because the deletion mutant was expressed on the surface of these cells.

with 1 μg/ml of EGF or with buffer alone as a control. The EGF-R was immunoprecipitated with RK2 antiserum, and the washed immunoprecipitates were exposed to phosphorylation reaction mixture containing [γ^{32}P]ATP and $MnCl_2$. Quantitation of the autoradiograms shown in Figure 9 indicated that the addition of EGF enhanced autophosphorylation of wild type receptor by fivefold and of deletion mutants by twofold. This indicates that the binding affinity of EGF toward mutant receptors is sufficiently large to allow activation of the kinase function. However, the ability of EGF to stimulate the kinase of the mutant receptor is reduced as compared with wild type receptor. Unfortunately, it is impossible to use this assay to estimate EGF binding affinity, as oversaturating concentrations of EGF were required for optimal stimulation of the kinase activity of the wild type or native EGF-R.

Discussion

The binding of EGF to the extracellular portion of the EGF-R leads to the activation of the cytoplasmic kinase domain, which undergoes autophosphorylation and phosphorylates various cellular substrates (Ushiro and Cohen, 1980). The EGF-R represents a typical allosteric receptor, and its natural ligand EGF activates the cytoplasmic kinase function by inducing a transmembrane allosteric transition (reviewed in Schlessinger, 1986, 1988). The allosteric transition is probably mediated by subunit interaction between oligomeric receptors (Yarden and Schlessinger, 1985, 1987a,b; Schlessinger 1986, 1988; Boni-Schnetzler and Pilch, 1987), a mechanism common to many regulatory enzymes (reviewed in Fersht, 1985). Hence, to understand the mechanism of receptor activation in molecular terms, it is important to identify the ligand-binding region of the EGF-R and the nature of the ligand-induced allosteric transition that activates the cytoplasmic kinase function. Analysis of the properties of EGF-R deletion mutants provided new insights into the ligand-binding region of the extracellular portion of the EGF-R and also revealed the importance of receptor interactions in control of receptor traf-

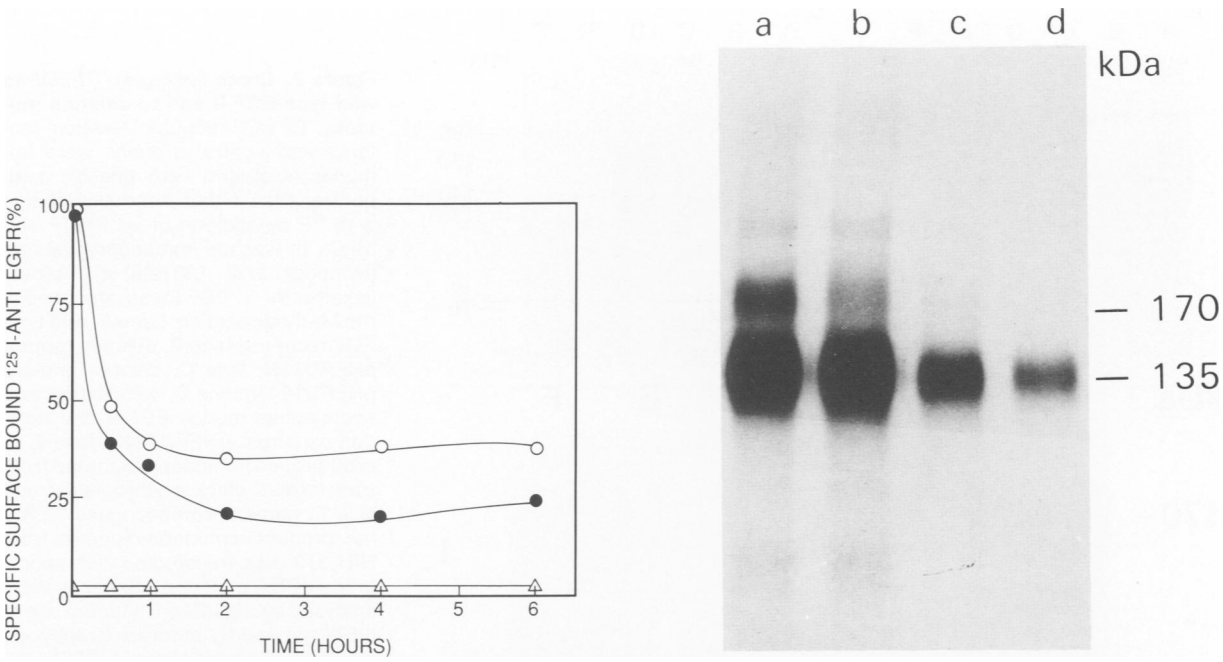

Figure 7. **EGF-receptor down regulation and degradation.** (A) Receptor down regulation. Cells were incubated with 500 ng/ml EGF at 37°C for different lengths of time. Thereafter, the cells were placed on ice and incubated for 2 h with ^{125}I-labeled-mAb108, which does not interfere with binding of EGF to the receptor and binds to human but not to murine EGF-R. The relative amount of ^{125}I-labeled mAb bound to cells is plotted against the time of incubation with EGF. NIH-3T3 cells that express deletion mutant (NpHERD124N cells) (△); NIH-3T3 cells that express wild type human EGF-R (HER1 cells) (○) and NIH-3T3 cells that express endogenous murine EGF-R and deletion mutant, NpHERD124NI cells (●). (B) Receptor degradation. NIH-3T3 cells that express endogenous murine EGF-R and deletion mutant (NpHERD124NI cells) were incubated in the presence of 1 μg/ml of EGF for different lengths of time. Subsequently, the cells were lysed, subjected to immunoprecipitation with mAb108, internally labeled with ^{32}P, and analyzed by SDS-PAGE with the use of 6.5% gel and by autoradiography. Lane a, 0 min; lane b, 30 min; lane c, 1 h; and lane d, 6 h.

ficking. These two topics will be discussed in this manuscript.

Evidence for EGF-R oligomerization and its role in surface expression

The binding of EGF to its receptor induces EGF-R oligomerization both in vitro and in living cells (Zidovetzki *et al.,* 1981, 1986; Hillman and Schlessinger, 1982; Schlessinger *et al.,* 1978; Haigler *et al.,* 1978; Yarden and Schlessinger, 1985, 1987a,b; Cochet *et al.,* 1988). EGF-induced receptor oligomerization provides an allosteric mechanism for the activation of the cytoplasmic kinase function by receptor-receptor interactions that are initiated at the cell surface, leading to kinase activation and biological responsiveness (Yarden and Schlessinger, 1985, 1987a,b; Schlessinger, 1986, 1988; Boni-Schnetzler and Pilch, 1987; Shechter *et al.,* 1979). Receptor oligomerization not only activates the kinase function, it also allows receptor autophosphorylation to proceed by an intermolecular process both in vitro and in living cells (Honegger *et al.,* 1989). However, the kinase activity of EGF-R can be regulated by ligand-independent mechanisms that do not involve EGF-R oligomerization (Koland and Cerione, 1988; Northwood and Davis, 1988).

In this report we provide further evidence for specific interactions between EGF-R molecules, indicating that receptor oligomerization may occur even in the absence of ligand binding. We have shown that it is possible to immunoprecipitate murine EGF-R with the use of human specific anti-receptor antibodies from lysates of transfected cells that express both endogenous murine and mutant human EGF-R. This association is maintained during the process of receptor solubilization and immunoprecipitation, indicating that the receptor molecules are held together by relatively strong forces and that domains other than domain I are essential for this interaction. This microscopic association, demonstrated by immunoprecipitation experiments, appears to have an interesting cellular correlate. The mutant receptors failed to be transported to the cell surface unless the transfected cells

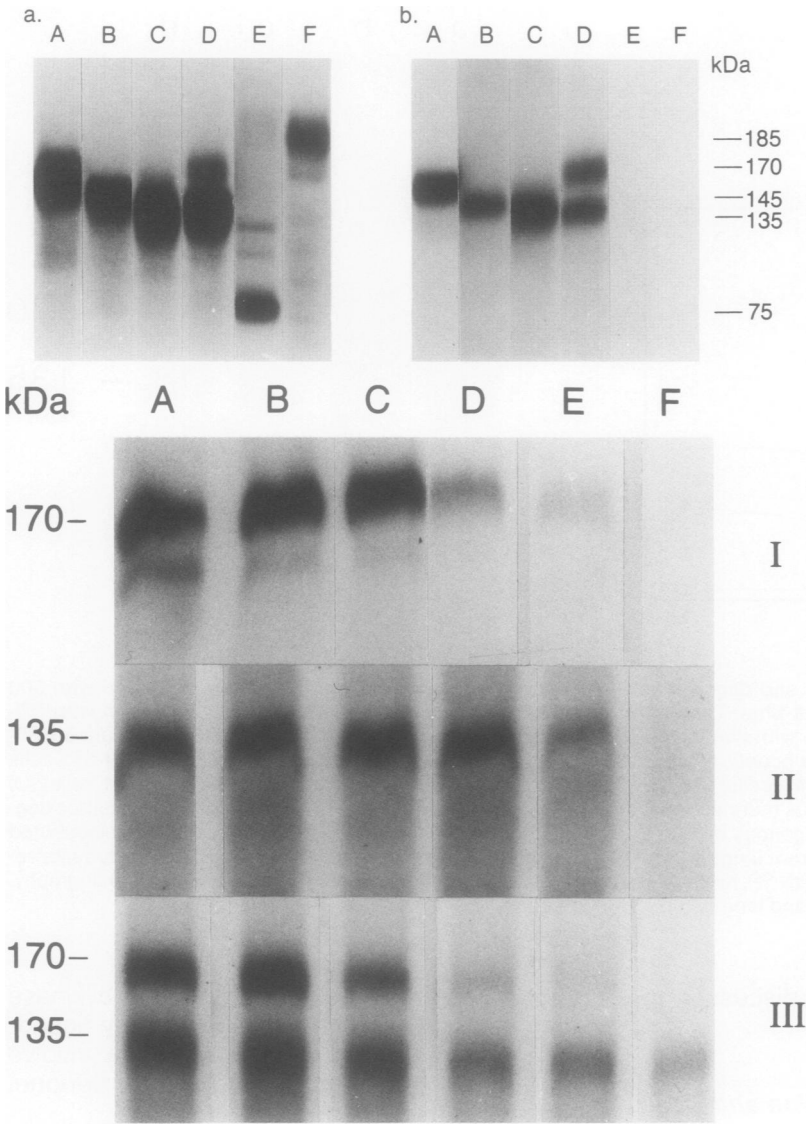

Figure 8. **Cross-linking of ^{125}I-EGF to wild type EGF-R and to deletion mutants.** (I) EGF-receptor deletion mutants and control proteins were immunoprecipitated with specific antibodies, then either internally labeled with ^{32}P by addition of [γ^{32}P]ATP and $MnCl_2$ to washed immunoprecipitates (Honegger *et al.,* 1987a,b) (a) or cross-linked with ^{125}I-EGF (b) as described in the Methods section. Lane A, wild type EGF-receptor; lane B, deletion mutant pHERD76N; lane C, deletion mutant pHERD124N; lane D, cells expressing endogenous murine EGF-R and deletion construct pHERD124N; lane E, v-erbB protein immunoprecipitated from transformed chick erythroblasts; and lane F, human protooncogene HER2/*neu* product immunoprecipitated from NIH-3T3 cells transfected with appropriate cDNA construct. Samples were analyzed by SDS-PAGE with the use of 6.5% gel and by autoradiography. (II) Inhibition of cross-linking of ^{125}I-EGF by native EGF. Wild type and mutant EGF-R were immunoprecipitated utilizing RK2 anti-EGF-R antibodies. Then increasing concentrations of native EGF were added to the reaction mixture for covalent cross-linking containing ^{125}I-EGF as described in the Methods section. After five washes the samples were analyzed by SDS-PAGE with the use of 6.5% gel and autoradiography. I, Wild type EGF-R, pHER; II, deletion mutant pHERD76N; and III, immunoprecipitates from cells expressing both endogenous murine EGF-R and deletion mutant (NpHERD124NI cells). Lane A, no EGF; lane B, 50 ng/ml EGF; lane C, 0.5 μg/ml EGF; lane D, 5 μg/ml EGF; lane E, 50 μg/ml EGF; and lane F, 500 μg/ml EGF.

contained also native EGF-R molecules. We interpret this result as an indication that the newly synthesized mutant receptors are trapped in the endoplasmic reticulum. It is now well established that many membrane proteins are retained in the endoplasmic reticulum when mutated (Rose and Bergmann, 1982, 1983; Wills *et al.,* 1984; Garoff *et al.,* 1983; Doyle *et al.,* 1985, 1986; Zuniga and Hood, 1986; Hardwick *et al.,* 1986). The retention of these proteins is probably due to improper folding and assembly and may also involve binding to an endoplasmic reticulum–binding protein called Bip (Morrison and Scharff, 1975; Haas and Wabl, 1983; Bole *et al.,* 1986; Gething *et al.,* 1986). However, once interaction with native receptors is established, this defect is "repaired" and the olibomeric complex containing both native and mutant receptors is normally delivered to the cell surface. This result may also indicate that receptor oligomerization plays a role in the trafficking of newly synthesized receptor from cytoplasmic compartment to the cell surface. Indeed, oligomerization appears to be essential for the transport of vesicular stromatitis viral glycoprotein to the cell surface (Kreis and Lodish, 1986; Copeland *et al.,* 1986). We have estimated the amounts of mutant and native receptors that are expressed on the cell surface utilizing binding experiments with human specific monoclonal anti-EGF-R antibodies and ^{125}I-EGF as specific probes. The transfected cells that express both species display on their surface 35 000 ± 5000 (SD) native murine EGF-R and

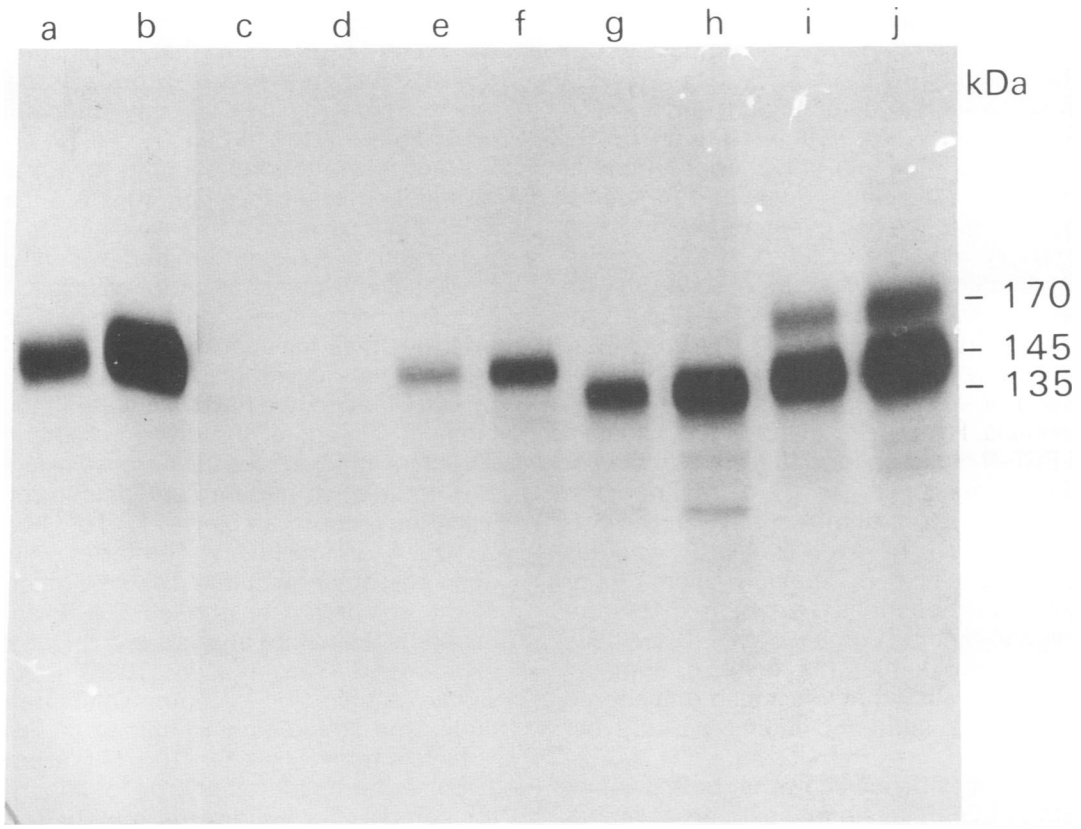

Figure 9. **Autophosphorylation of immunoprecipitated wild type EGF-R and deletion mutants.** Lysates of cells expressing wild type EGF-R and deletion mutants were immunoprecipitated with RK-2 anti-EGF-R antibodies, then 1 μg/ml EGF was added for 20 min at 37°C followed by phosphorylation reaction mixture containing 5 μM [γ^{32}P]ATP as described in the Methods section. Samples were analyzed by SDS-PAGE with the use of 6.5% gel and autoradiography. Lanes a and b, NIH-3T3 cells expressing wild type EGF-R (HER1 cells); lanes c and d, parental NIH-3T3 cells; lanes e and f, NIH-3T3 cells expressing deletion mutant (NpHERD76N cells); lanes g and h, NIH-3T3 cells expressing deletion mutant (NpHERD124N cells); and lanes i and j, NIH-3T3 cells expressing endogenous murine EGF-R and deletion mutant (NpHERD124NI cells). Lanes b, d, f, h, and j show the effect of EGF on the autophosphorylation of EGF-R and autophosphorylation of deletion mutants.

100 000 ± 35 000 (SD) human mutant EGF-R. This may suggest that each native receptor is able to facilitate surface transport of two to three mutant receptors, probably by forming hybrid oligomers between mouse and human EGF-Rs. However, these are not precise measurements, and the determination of the stoichiometry between endogenous and mutant EGF-Rs requires more rigorous analysis.

Once the mutant receptor is expressed on the cell surface, EGF is able to "down regulate" and also induce the degradation of mutant receptors. Although not displayed on the cell surface, the biosynthetic half-life of the mutant receptor is similar to the half-life of wild type or native EGF-R (10–12 h). However, the half-life of both native and mutant EGF-R was decreased to ~1 h when EGF was added to cells that display on their surfaces both endogenous and mutant EGF-R (Stoscheck and Carpenter, 1984; Honegger *et al.,* 1987a,b). It is not clear yet whether the binding affinity of mutant receptors is large enough to drive receptor internalization and degradation. An alternative explanation is that EGF induces the down regulation and degradation only of native receptors and that the mutant receptors, which are associated with the native molecules, follow their fate.

Ligand-binding specificity of deletion mutants

The extracellular portion of the mature EGF-R contains two cysteine-rich domains (Ullrich *et al.,* 1984). It is possible to arbitrarily divide the extracellular portion to four separate domains, each composed of 120–150 amino acid residues (Figure 1). Domain I spans the region from the

amino terminal residue up to the first cysteine-rich region, which is defined as domain II. Domain III is flanked by the first cysteine-rich domain and the second cysteine-rich region, which we call domain IV. We speculate that each of these domains may fold independently and is large enough to individually constitute a ligand-binding domain. The two cysteine-rich domains are probably too rigid to function as a ligand-binding domain. Moreover, other receptors with different ligand specificities, such as insulin receptor (Ullrich *et al.,* 1986; Ebina 1986) or HER2/neu oncogene product (Schechter *et al.,* 1984; Coussens *et al.,* 1985), have similar cysteine-rich domains. Hence, the ligand-binding domain of the EGF-R could be formed from domain I or domain III individually or by a configuration composed of all four domains in which the cysteine-rich domains have a skeletal role, whereas the ligand specificity is defined by domain I or III or by their interactions. To explore the nature of the ligand-binding domain of EGF-R, we have generated deletion mutants devoid of domain I or part of domain I that were also engineered to contain the authentic signal peptide sequence.

Two lines of evidence show that both deletion mutants of EGF-R retain at least partial ligand-binding capacity. It is possible to covalently cross-link ^{125}I-EGF to both mutant receptors, and the cross-linking reaction is specific inasmuch as native EGF or monoclonal anti-EGF-R antibodies (which compete for ligand binding) block the cross-linking reaction of ^{125}I-EGF to wild type, native, and mutant receptors. Moreover, a related receptor molecule (HER2/*neu*) and EGF-R protein with a deletion of most of the extracellular portion (v-erbB) do not undergo covalent cross-linking with ^{125}I-EGF, further demonstrating the specificity of the cross-linking reaction. The second manifestation of ligand binding is the ability of EGF to stimulate, albeit weakly, the autophosphorylation of both deletion mutants, indicating that EGF is indeed bound to its receptor—otherwise it would not activate the protein tyrosine kinase of mutant molecules.

The ligand dissociation constant (K_d) is usually deduced from analysis of binding experiments of radiolabeled ligand according to the method of Scatchard (1949). This was difficult to accomplish for the mutant receptors for several reasons. We have shown that the NIH-3T3 cells that express these mutants do not display them on the cell surface unless the transfected cells also contain native EGF-Rs. Moreover, ^{125}I-EGF binding experiments to transfected NIH-3T3 cells that display on their surface both native and mutant EGF-R show that the binding affinity of EGF toward mutant receptors is probably too low to be determined by conventional binding experiments with ^{125}I-EGF.

What is the theoretical limit for reliable determination of dissociation constant from ^{125}I-EGF binding experiments? We estimate that the upper limit of K_d as determined by conventional binding experiments to intact cells is $K_d \leq 10^{-7}$ M.† This dissociation constant is probably sufficiently small for specific cross-linking of ^{125}I-EGF and for stimulation of EGF-R kinase activity by EGF. Indeed, we have calculated the ligand-binding affinities of the deletion mutants from ID_{50} of inhibition of covalent cross-linking of ^{125}I-EGF to deletion mutants and as control to the wild type receptor to be $(1–2) \times 10^{-7}$ M and 1.3×10^{-8} M, respectively. The latter value is in good agreement with the dissociation constant of the solubilized or purified EGF-R as determined by Scatchard analysis of ^{125}I-EGF binding experiments (Yarden *et al.,* 1985). It was previously shown that receptor solubilization reduces the ligand-binding affinity of EGF-R by ~10-fold from $K_d = 1.5 \times 10^{-9}$ M (Yarden *et al.,* 1985). In terms of free energy for ligand-receptor complex formation, the mutant receptors retained 85% of the free energy of association for EGF and EGF-R (−9.3 kcal/mol as compared with −10.8 kcal/mol for wild type EGF-R). This implies that, in spite of deletion of domain I, most of the binding energy for EGF is retained in the mutant receptor. In a separate line of experiments, we have affinity-labeled the EGF-R by the use of covalent cross-linking agents and ^{125}I-EGF as a probe. Utilizing site-specific anti-peptide antibodies, we have isolated from the labeled receptor an ^{125}I-EGF–containing fragment that encompasses domain III of EGF-R (Lax *et al.,* 1988a). These results are consistent with the notion that domain III contributes most of the interactions between EGF and EGF-R. Moreover, we have also described the binding properties toward EGF of chicken/human chimeric EGF-R molecules expressed in NIH-3T3 cells and shown that domain III of EGF-R is a major ligand-binding domain (Lax *et al.,* 1989). Further analysis of additional chicken/human chimer of EGF-R indicated that, although domain III is a ligand-binding region, domain I also contributes at least part of the interactions that determine binding specificity toward EGF (Lax *et al.,* submitted). We suggest that domain III, which is flanked by the two cysteine-rich domains, contributes most of the forces that define EGF-binding specificity. The remaining free

energy (−1.5 kcal/mol) is probably gained by additional interactions between EGF and domain I or between EGF and the unperturbed domain III within the naturally folded suprastructure. It appears that EGF is able to interact with domains III and I, and therefore the ligand-binding region may lie in the cleft formed between these two domains (Figure 10). Such a configuration for ligand-binding region is common in many allosteric enzymes (reviewed in Fersht, 1985), where ligand binding alters the interaction between neighboring subunits, thus allowing the transfer of an allosteric conformational transition.

In summary, our experiments suggest that domain III, which is flanked by the two cysteine-rich domains, contributes most of the binding forces for the interaction between EGF and EGF-R. On the basis of this result, we propose a four-domain model for the organization of the extracellular portion of the EGF-R and for the localization of the EGF-binding region. We have demonstrated, in addition, a specific microscopic interaction between mutant and native EGF-R, which suggests that EGF-R molecules exist in the plane of the membrane as oligomers even in the absence of ligand binding. This receptor interaction can complement the mutation as far as trafficking to the cell surface is concerned and may, therefore, play a role in the process of normal transport of EGF-R to the cell surface.

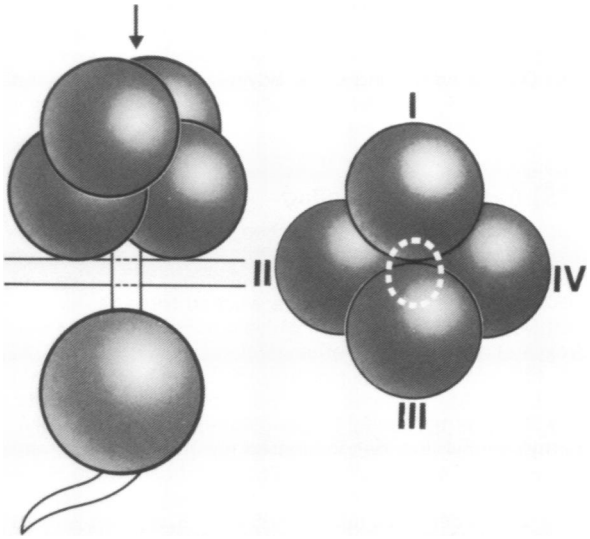

Figure 10. **A four-domain model for the extracellular portion of the EGF-R.** Individual spheres correspond to the four domains (I–IV) shown in the linear model presented in Figure 1. Left, side view; right, top view. Arrow and dashed line show the putative ligand binding region, which is postulated to be in the cleft formed between domain III and domain I. It is proposed that domain III contributes most of the ligand binding activity. More details concerning this model are provided in the Discussion.

Methods

Construction of deletion mutants

To construct deletion mutants in the cDNA of the EGF-R, the 5′ 1.17 kb *Xho* I-*Bam*HI fragment of the cDNA was isolated from pLSX (Livneh *et al.,* 1986a,b) and subcloned into pAZ1037 (Schmidt *et al.,* 1985) to generate HERf 1.2 (Figure 1). This plasmid was first cut with BanII (codons 23–24 of EGF-R), blunted with T_4 polymerase and then digested with *Eco*RI (codons 98–99 of EGF-R) and again with filled-in klenow DNA polymerase. The linearized plasmid was purified by gel electrophoresis followed by ligation of the two blunt ends to generate HERf 1.0 (Figure 1), with a deletion of 76 codons between codons 23 and 99. To generate HERf 0.8 (Figure 1), the HERf 1.2 was cut with BanII and treated with T_4 polymerase followed by digestion with *Nar* I (codon 146 of EGF-R). Self-ligation of the isolated plasmid generated HERf 0.8 (Figure 1), with a deletion of 124 codons between codons 23 and 147. The deletions and the conservation of the reading frame of HERf 1.0 and HERf 0.8 were confirmed by DNA sequencing (Maxam and Gilbert, 1977).

The deletion-containing fragments were isolated from either HERf 1.0 or HERf 0.8 by cutting with *Xma* III (codon 6) and *Xma* I (codon 243) and separating the insert on Agarose gel. The isolated fragments were reinserted into the unique *Xma* III and *Xma* I site of pLSX. (The *Xma* III site in the pBR 322 portion of pLSX was eliminated by cutting with *Sal* I and *Nru* I followed by religation). The resulting plasmids (Figure 1B) pHERD76N (with deletion of 76 codons) and pHERD124N (with deletion of 124 codons) were grown in *E. coli* HB101, and plasmid DNA was isolated on CsCl gradient.

Transfections

NIH-3T3 cells (done 2.2) were grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) with 10% fetal calf serum. Dishes of cells (10 cm) were transfected with 10–20 μg of plasmid -DNA/dish by the use of the calcium/phosphate precipitation technique (Wigler *et al.,* 1979). Two days after transfection, the cells were split, seeded at a density of 100 000 cells/10-cm dish, and put under neomycin-resistance selection by adding 0.8 mg/ml of Geneticin G418 (GIBCO) to the medium. Resistant clones were picked after 3 wk and screened by immunoprecipitation of phosphorylated or [^{35}S]methionine-labeled receptor with various antibodies against EGF-R.

^{125}I-EGF binding experiments

For all ^{125}I-EGF binding assays, cells were seeded at a density of 100 000 cells/well in 24-well dishes coated with 10 μg/well of human plasma fibronectin (Rorer Biotechnology Inc.) and allowed to grow for 48 h to confluency in DMEM containing 10% fetal calf serum. Mouse EGF (I.D.L., Jerusalem) was iodinated, with the use of the chloramin-T method, to a specific activity of 100 000–200 000 cpm/ng.

Receptor down regulation

Confluent cells growing on fibronectin-coated 6-well Costar dishes were incubated with 1 μg/ml of unlabeled EGF for up to 4 h at 37°C. After washing of EGF, the cells were

incubated with ^{125}I-labeled purified IgG 108.1 (monoclonal antibody against the extracellular domain of the human EGF-R) for 2 h at 4°C. Then the cells were washed three times with DMEM containing 0.1% bovine serum albumin and lysed with 0.5 N NaOH for 30 min at 37°C to determine their radioactive content.

[^{35}S]methionine labeling

Subconfluent cells in 10-cm dishes were washed with methionine-free DMEM and grown for 12 h in methionine-free DMEM/10% fetal calf serum containing 100 μCi/ml of [^{35}S]methionine. The cells were washed three times with DMEM; scraped into 0.5 ml of lysis buffer (20 mM 4-(hydroxymethyl)-piperazine-ethanesulfonic acid [HEPES] pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM [ethylenebis (oxyethylenenitrilo)]tetraacetic acid, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride); incubated for 30 min on ice; and the lysate spun for 5 min in an Eppendorf centrifuge in the cold. Three milligrams of protein A-Sepharose per sample were suspended in 20 mM HEPES pH 7.5, washed with 20 mM HEPES and incubated for 30 min at room temperature with monoclonal antibody IgG 108.1 or with antiserum RK2. The protein A-Sepharose-antibody complex was washed three times with HNTG (20 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100) and incubated with the cell lysate for 90 min at 4°C. The immunoprecipitate was then washed twice with 50 mM HEPES pH 8.0, 0.2% Triton X-100, and 500 mM [ethylenebis (oxyethylenenitrilo)]tetraacetic acid; twice with 50 mM HEPES pH 8.0, 0.1% Triton X-100, 0.1% SDS, 150 mM NaCl, and 5 mM [ethylenebis (oxyethylenenitrilo)]tetraacetic acid; and twice with 10 mM Tris/HCl pH 8.0 and 1.0% Triton X-100. Then three volumes of sample buffer were added to the washed immunoprecipitate, boiled for 4 min, and electrophoretically separated on a 6.5% SDS-polyacrylamide gel.

Receptor degradation

Cells labeled for 12 h with [^{35}S]methionine were incubated for increasing periods with or without 1 μg/ml of EGF in serum-free DMEM before lysis and immunoprecipitation as described above.

Phosphorylation experiments

Dishes of confluent cells were washed three times with phosphate-buffered saline, drained well, and scraped into 0.5 ml of lysis buffer. After 30 min incubation on ice, the lysate was centrifuged for 5 min in an Eppendorf centrifuge in the cold and the supernatant immediately used or kept frozen at −70°C. Lysates were incubated with protein A-Sepharose-antibody complex for 90 min in the cold, then washed two times with HNTG containing 500 mM NaCl and three times with HNTG containing 150 mM NaCl. For autophosphorylation, 30 μl of HNTG containing 15 mM $MnCl_2$, 200 μM sodium orthovanadate, 3 μCi of [γ^{32}P]ATP and 15 μM of unlabeled ATP were added to the immunoprecipitate and incubated for 1 min on ice. The reaction was stopped by addition of 20 μl of sample buffer (3X) and boiling for 4 min. The proteins were separated by 6.5% SDS-PAGE.

Cross-linking experiments with ^{125}I-EGF

Dishes containing confluent cells were washed with phosphate-buffered saline, drained well, and scraped into 0.5 ml of lysis buffer. After 30 min on ice, the lysates were centrifuged for 5 min in an Eppendorf centrifuge in the cold, and the supernatants were incubated with protein-A sepharose RK2 antibody complex for 90 min in the cold. The immunoprecipitates were washed five times with HNTG buffer and incubated with 5 μg/ml of ^{125}I-EGF (final volume 50 μl) for 30 min at 37°C. For the determination of ID_{50} for the inhibition of covalent cross-linking of ^{125}I-EGF, the samples were incubated with, in addition to ^{125}I-EGF, various concentrations of unlabeled EGF; then analyzed by SDS-PAGE; and quantitated by densitometric analysis of the autoradiograms. Subsequently, three volumes of sample buffer were added to the washed immunoprecipitates, and the samples were boiled for 4 min. The proteins were separated by electrophoresis on 6.5% SDS-polyacrylamide gel.

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