

Large Deletions in the Cytoplasmic Kinase Domain of the Epidermal Growth Factor Receptor Do Not Affect Its Lateral Mobility

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Abstract. The lateral diffusion coefficients of various epidermal growth factor (EGF) receptor mutants with increasing deletions in their carboxy-terminal cytoplasmic domain were compared. A full size cDNA construct of human EGF receptor and different deletion constructs were expressed in monkey COS cells. The EGF receptor mutants expressed on the cell surface of the COS cells were labeled with rhodamine-EGF, and the lateral diffusion coefficients of the labeled receptors were determined by the fluorescence photo-

bleaching recovery method.

The lateral mobilities of three deletion mutants, including a mutant that has only nine amino acids in the cytoplasmic domain, are all similar ($D \cong 1.5 \times 10^{-10}$ cm²/s) to the lateral mobility of the "wild-type" receptor, which possesses 542 cytoplasmic amino acids. It is concluded that most of the cytoplasmic domain of EGF receptor, including its intrinsic protein kinase activity and phosphorylation state, are not required for the restriction of its lateral mobility.

EPIDERMAL growth factor (EGF)¹ is a small protein that acts as a mitogen for various cell types bearing specific EGF receptors (reviewed in references 3 and 21). The EGF receptor molecule is a glycoprotein of 170,000 mol wt (3, 21) that is composed of three functional domains: an extracellular EGF-binding domain, a transmembrane region, and a cytoplasmic kinase domain (26). EGF binds to randomly dispersed receptors that rapidly cluster in the region of membrane coated pits and then becomes internalized, delivering the occupied receptors to lysosomes where both EGF and the receptor become degraded by proteolytic enzymes (3, 4, 21). The clustering and internalization of EGF receptor complex on cultured cells are temperature-sensitive processes that are completely inhibited at 4°C.

Comparison of the lateral and rotational diffusion of the EGF receptor indicates that the rotational diffusion of the EGF receptor is controlled by the viscosity of the lipid matrix (30), whereas the lateral diffusion of the EGF receptor seems to be limited by interactions in addition to viscous drag (11). The lateral diffusion of EGF receptor in the membrane of human A-431 cells and mouse 3T3 cells is $1.5\text{--}0.3 \times 10^{-10}$ cm²/s (11, 21). This value is similar to the diffusion coefficient measured for many membrane proteins (reviewed in references 14 and 22). The additional interactions that limit the lateral diffusion of EGF receptor specifically as well as many other membrane proteins, in general, could be due to interactions with the underlying cytoskeletal network, interactions with other membrane proteins, or interactions with the extracellular matrix.

1. *Abbreviations used in this paper:* DME, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; FPR, fluorescence photobleaching recovery; Rh-EGF, rhodamine-EGF; SV-40, simian virus 40.

In this study we compare the lateral diffusion of various EGF receptor mutants with increasing deletions in their carboxy-terminal cytoplasmic kinase domain composed of 542 amino acids (26). A full-sized cDNA construct of the EGF receptor and different deletion constructs were expressed in monkey COS cells. The COS cells are CV-1 monkey kidney cells transformed by a simian virus 40 (SV-40) genome defective in the origin of replication (10). Since COS cells constitutively express the large T antigen, transfected plasmids containing an SV-40 origin can replicate to high copy numbers and yield high amounts of protein.

The study of lateral mobilities in the COS cell system has been advantageous since: (a) Analysis is fast and protein products can be analyzed 2–3 d after transfections. (b) The COS cells express large quantities of EGF receptor and its mutants. Moreover, similar amounts of receptor and its mutants are expressed in the transfected cells. This is in contrast to stable cell lines obtained by transfection, which usually express variable amounts of protein products. (c) The lateral diffusion coefficients of the EGF receptor and its various mutants labeled with rhodamine EGF (Rh-EGF) are measured using the fluorescence photobleaching recovery (FPR) method (reviewed in references 14 and 22). In FPR, individual cells are analyzed; therefore, the fact that only 5–10% of the COS cells express the various EGF receptor cDNA constructs is not a limitation.

Our results show that large deletions in the cytoplasmic domain of the EGF receptor do not affect its lateral mobility and even a mutant that has only nine amino acids in the cytoplasmic domain still translates with an apparent diffusion coefficient of $D \cong 1.5 \times 10^{-10}$ cm²/s. It is concluded that most of the cytoplasmic domain of EGF receptor, in-

cluding the integrity of the receptor kinase activity and of various phosphorylation sites, are not required for the restriction of the lateral mobility of the EGF receptor.

Materials and Methods

Cells

Monkey COS cell (10) clone M6, kindly provided by M. Horowitz (Weizmann Institute), and human A-431 cells were grown in Dulbecco's modified Eagle's medium (DME) containing 10% fetal calf serum, supplemented with penicillin-streptomycin. For microscopic studies, the cells were grown on glass coverslips in 35- or 100-mm tissue culture dishes.

Recombinant Plasmids

The full length cDNA was pieced together from overlapping lambda phage cDNA clones λ HER-A64 and λ HER-A21 (26) and subcloned into PBR322. The final subclone extended from the SstI site, 15 base pairs (bp) before the initiation codon for translation (nucleotide 171 according to Ullrich et al. [26]), to an XmnI site 151 bp after the termination codon (nucleotide 3,970). Synthetic XhoI linkers DNA were used to convert both ends to XhoI sites and were then subcloned into the Pvu II site of PBR322 that was changed similarly to an XhoI site. The EGF receptor cDNA with XhoI ends was then placed into an early substitution SV-40 based expression vector. This vector is similar to pLSV (17) except that a polylinker containing a BglIII and an SmaI site was introduced between the HindIII and the BclI sites. This plasmid was generously provided by O. Laub (Weizmann Institute). The SmaI site was changed to an XhoI site and was used for cloning the plasmid. The final plasmid in the correct orientation denoted as pLSX is diagrammed in Fig. 1.

The plasmid pLSXC was constructed from the plasmid pLSX. pLSX was digested with HincII, and XhoI linkers were added to the fragments. Excess digestion with XhoI released a 3.6-kb fragment that, after gel purification, was ligated into the XhoI site of the vector.

The plasmid p Δ B71 was constructed by partial digestion of pLSX with BalI. XbaI DNA linkers were added, and excess linkers were removed by digestion with XbaI followed by agarose gel electrophoresis. Purified fragments were self ligated to close the circle.

The plasmid p Δ NA8 was constructed from the p Δ B71 plasmid. p Δ B71 was digested by NarI and the staggered ends were filled in with a Klenow fragment of DNA polymerase I. The blunt-ended fragment was ligated with a synthetic 26-bp double-stranded oligonucleotide composed of 18 bp representing sequence 2,199-2,217 of the EGF receptor attached to a 7-bp sequence containing the XbaI site, with the following sequence: 5'-CCACATCGTTCGGAAGCCTCTAGAG-3'.

The ligated fragment was cleaved by SmaI and excess XbaI and was purified by gel electrophoresis. This 1.2-kb fragment was further ligated to an 8-kb SmaI-XbaI fragment, which was recovered from pLSX.

Each of the plasmids constructed was used to transform *E. coli* HB101, and the plasmids containing the cDNA constructs were tested for the presence of the desired deletions by digestion with appropriate restriction endonucleases. Enzymes were provided by New England Biolabs (Beverly, MA) and Boehringer-Mannheim Biochemicals (Indianapolis, IN) and were used as described by the manufacturers.

Transfection of COS Cells and Analysis of Expressed cDNA Constructs

Transfection experiments were performed according to the DEAE-dextran procedure as described by Sompayrac and Donna (24) with some modifications. COS cells from a confluent tissue culture dish were split (1:5) 2 d before transfection into tissue culture dishes containing glass coverslips. Shortly before transfection, the cells were washed with DME and incubated with 5 ml of DME containing 50 mM Tris-chloride (pH 7.3), 0.2 mg of DEAE-dextran per ml (2×10^6 mol wt, Pharmacia Fine Chemicals, Piscataway, NJ), and 10 μ g of plasmid DNA. After 6-8 h, the cells were washed extensively and DME containing 10% fetal calf serum was added. The cells were analyzed for the expression of the various EGF receptor constructs, 48-72 h after transfection. Cells were labeled with [35 S]methionine (50 μ Ci/ml) in methionine-free DME for 4-5 h, and cell extracts were prepared as previously described (16). Specific antibodies against EGF receptor coupled to Sepharose-protein A were used to immunoprecipitate the EGF receptor protein and its various deleted forms. The samples were analyzed

by gel electrophoresis on a 5-10% polyacrylamide gel containing SDS. The gels were treated with Amplify for 30 min, dried, and exposed for 18 h at -70°C for autoradiography. The autophosphorylation reaction of EGF receptor and its various mutants was performed according to the procedure described by Kris et al. (16).

The antisera that were used are as follows: RK-2 is a rabbit antiserum against a synthetic peptide from the carboxy-terminal region of the EGF receptor (16). Anti-AI is a rabbit antiserum prepared against A-431 cell membranes and reacts predominantly with the extracellular domain of the EGF receptor (29).

FPR

The lateral diffusion coefficient of the fluorescently labeled EGF receptor was measured by the FPR method (reviewed in references 14 and 22). In FPR, a small region on the cell surface is illuminated with a focussed attenuated laser beam (argon, 514 nm). Then, a brief (~ 100 ms) intense laser pulse irreversibly bleached the illuminated region, and the time course of recovery of fluorescence in the bleached region due to replenishment by unbleached fluorophores from adjacent regions on the cell surface was recorded. The fluorescence recovery curves were fitted to the full series solution of the diffusion equation (1) by a computer program that used a nonlinear least squares fitting procedure to estimate the best values for the lateral diffusion coefficient (D , cm^2/s) and the fraction of mobile labeled molecules on the cell surface ($R\%$) (19). All measurements were performed at room temperature.

Results

Various constructs of the EGF receptor cDNA with increasing deletions at the 3' coding region of the EGF receptor cDNA were prepared. The SV-40 early promoter was used

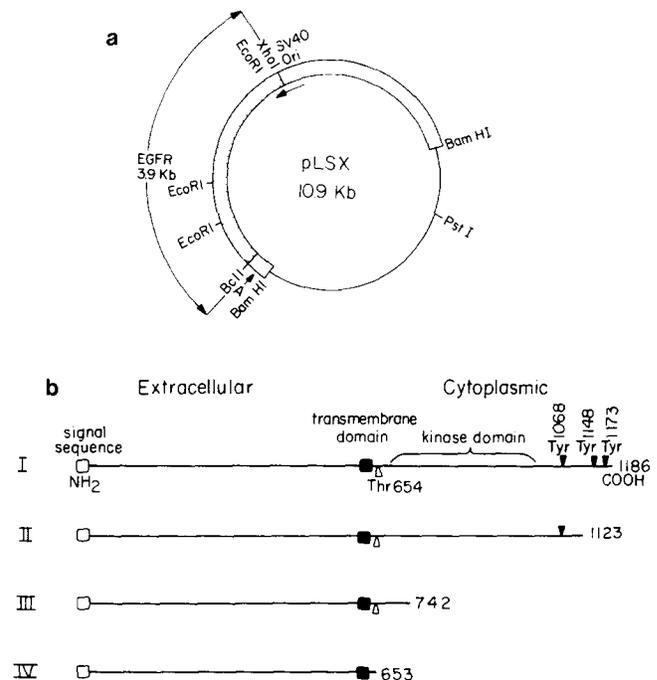


Figure 1. Structure of EGF receptor expression construct and mutants. (a) The structure of plasmid pLSX is shown in the right orientation. Thin line, PBR322 sequences; stippled bars, SV-40 sequences; and striped bar, EGF-receptor sequences. Ori, the SV-40 origin of replication. Arrow, the direction of transcription initiating at the SV-40 early promoter. (b) The structure of the coding region of the various EGF receptor mutants. Plasmids were constructed as described in the text. The signal sequence, the transmembrane domain, and the kinase domain are indicated. The amino acid numbering is as described in reference 26. Black arrows, positions of the tyrosine phosphorylation sites. (Thr 654) Open arrows, threonine phosphorylation sites.

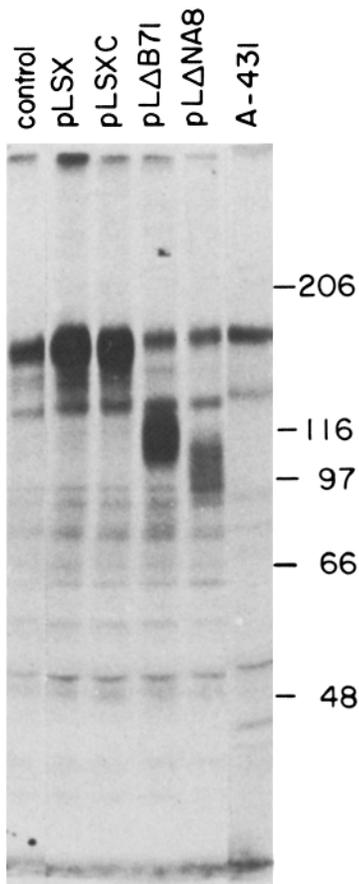


Figure 2. Analysis of biosynthetically labeled EGF receptor mutants expressed in the COS cells. Subconfluent cells growing in 10-cm dishes transfected with 10 μ g per dish of the various plasmids using the DEAE-dextran procedure. Control cells were transfected with 10 μ g of the same plasmid containing the dihydrofolate reductase coding sequence, or were mock transfected. The cells were labeled for 4 h, then solubilized and immunoprecipitated as described in Materials and Methods. Equal amounts of TCA-insoluble radioactivity were reacted with antibody anti-AI. Anti-AI is directed primarily against extracellular sequences of the EGF receptor. The positions of known molecular weight markers are indicated on the right in kilodaltons.

to transiently express the various constructs in COS cells (25). Fig. 1 shows the structure of the various constructs that were used in this study. (a) pLSX. This construct encodes the entire EGF receptor (*I* in Fig. 1 *b*). (b) pLSXC. A deletion of 63 amino acids at the carboxy tail terminating at residue 1,123 yields a receptor mutant devoid of two autophosphorylation sites (*II* in Fig. 1 *b*). (c) pL Δ B71. A large deletion at the cytoplasmic domain yields a receptor mutant devoid of most of the tyrosine kinase domain possessing 98 cytoplasmic amino acids (*III* in Fig. 1 *b*). (d) pL Δ NA8. This construct encodes an EGF receptor mutant with a small cytoplasmic region of nine amino acids terminating in residue 653. This mutant is devoid of Thr 654 which is the major kinase C phosphorylation site (*IV* in Fig. 1 *b*).

To analyze the sizes of the proteins expressed by the various EGF receptor cDNA constructs, the transfected COS cells were metabolically labeled with [35 S]methionine and then subjected to immunoprecipitation experiments with antibodies directed against the extracellular portion of the EGF

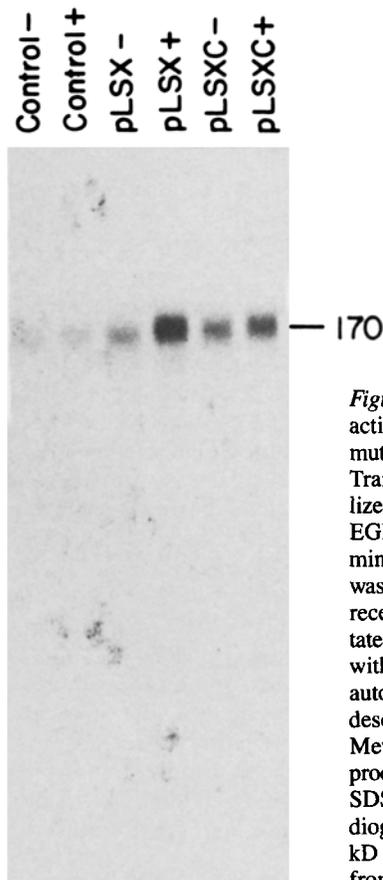


Figure 3. Analysis of the kinase activity of the EGF receptor mutants expressed in COS cells. Transfected cells were solubilized and treated with 5 μ g/ml EGF or with buffer alone for 20 min on ice. RK-2 antiserum was used to precipitate the EGF receptor. The immunoprecipitated receptor was incubated with [γ - 32 P]ATP in a standard autophosphorylation assay as described in Materials and Methods. The phosphorylated products were analyzed by 7.5% SDS PAGE followed by autoradiography. The position of 170 kD is shown, as determined from known markers.

receptor AgAI. Fig. 2 shows an autoradiogram of SDS PAGE analysis of the immunoprecipitated polypeptides. Control experiments (Fig. 2) indicate that the COS cells express endogenous EGF receptors. From quantitative binding experiments with 125 I-EGF, it is concluded that the COS cells express \sim 50,000 receptors per cell. The cells transfected with the various constructs express, in addition to the endogenous receptor, large quantities of the human EGF receptor and its various mutants (Fig. 2). The pLSX construct, which contains the entire coding region of the EGF receptor, appears as a polypeptide of 170,000 D. pLSXC, which has a deletion of 63 amino acids at the carboxy terminal, encodes a polypeptide of similar apparent molecular weight as revealed by this gel system. The pL Δ B71 construct encodes a polypeptide of 115,000 D, and the pL Δ NA8 construct encodes a polypeptide of 105–110 kD. Note that \sim 35,000 of the apparent molecular weight of the various EGF receptor mutants is due to the carbohydrates that are attached to the extracellular EGF-binding domain of the EGF receptor molecule (21, 26).

The EGF receptor expressed in COS cells by the pLSX construct binds EGF, and its intrinsic protein tyrosine kinase activity (27) can be enhanced several folds in the presence of the growth factor leading to autophosphorylation (Fig. 3). The carboxy terminus-deleted construct, pLSXC, lacking two autophosphorylation sites (7), can still be autophosphorylated in the presence of EGF to some extent, probably by using other autophosphorylation sites.

After 48–72 h the transfected cells were incubated with Rh-EGF to detect cells that express the various EGF receptor mutants on their cell surface. Note that the COS cells express

~50,000 endogenous EGF receptors per cell. This amount of receptors can be barely visualized after the cells were labeled with Rh-EGF and is much lower than the fluorescent labeling of the COS cells that express the various EGF receptor constructs. Quantitative fluorescence measurements using the photomultiplier system of the FPR apparatus indicate that the COS cells that express the various EGF receptor mutants possess 50–100-fold more binding sites for Rh-EGF than the untransfected COS cells (Table I). As previously observed for the COS system, only 5–10% of the cells express large quantities of the various constructs (24).

The lateral diffusion of the various EGF receptor mutants expressed in the COS cells and labeled with Rh-EGF was measured by the FPR method (14, 22). The intensity of the fluorescence of Rh-EGF bound to the cells that bear the various EGF receptor mutants was similar (Table I), indicating that these mutants are expressed to similar levels on the cell surface. Table II summarizes the lateral diffusion coefficients of the various EGF receptor mutants. It appears that all the EGF receptor mutants translate in the membrane of the COS cells with a similar diffusion coefficient of $D \cong 1.5 \times 10^{-10}$ cm²/s at room temperature. The mobile fraction of the various mutants is also similar in the range of $R\% = 50$ – 80% . A similar value of D was measured for EGF receptor in A-431 cells at room temperature. Table II also gives the lateral diffusion of the fluorescent lipid probe nitrobenzoxadiazole-phosphatidylethanolamine in the membrane of the COS cells. D lipid probe = 4.4×10^{-9} cm²/s, a value similar to the diffusion coefficient of lipid probes measured in many cultured cells.

Discussion

The pleiotropic effects of EGF are mediated by the EGF receptor. The EGF receptor possesses intrinsic protein tyrosine kinase activity, which is regulated by the binding of EGF to the extracellular binding domain (3, 27). The EGF receptor kinase phosphorylates various cellular proteins and at least three tyrosine residues, which are located at the carboxy-terminal end of the EGF receptor itself (7). Moreover, kinase C activated by the tumor promoter ester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) phosphorylates Thr 654 (6, 12) of the receptor molecule, a residue located 10 amino acids in the cytoplasmic domain of the EGF receptor. Since TPA reduces the affinity of the receptor towards EGF (15, 23)

Table I. Quantitative Measurements of Fluorescence Intensity of COS Cells That Express EGF Receptor Mutants

| Cell type and construct | Fluorescence intensity |
|-------------------------|--------------------------------|
| | counts/s per 1 μm^2 |
| pLSX construct in COS | 5,000 \pm 1,500 |
| pLSXC construct in COS | 4,500 \pm 1,500 |
| pLAB71 construct in COS | 5,500 \pm 1,800 |
| pLANA8 construct in COS | 3,000 \pm 1,100 |
| Untransfected COS cells | 60 \pm 15 |
| A-431 cells | 900 \pm 150 |

All fluorescence measurements were performed with a laser beam (200 mW) attenuated 1/20,000. The various cells were labeled with Rh-EGF as described in Materials and Methods, and the fluorescence intensity was measured using the photomultiplier systems of the FPR apparatus. Values represent mean \pm SD.

and its tyrosine kinase activity (5, 13), it was proposed that the phosphorylation of Thr 654 may regulate these functions (6, 12). TPA also effects the internalization of the EGF receptor and therefore it is possible that the phosphorylation state of the receptor could play a role in its endocytic routing (2).

In this study we compare the effect of various deletions in the cytoplasmic kinase domain of the receptor on its dynamic properties. Our results show that all the mutants translate in the plane of the membrane with a similar diffusion coefficient, even though the truncations effected endocytosis drastically (18).² The degree of endocytosis of the pLSXC construct encoding a receptor with kinase activity but without two autophosphorylation sites is comparable to the full receptor. The EGF receptor mutant pLAB71 is devoid of kinase activity, and pLANA8 is further deleted to yield a mutant without Thr 654. Both mutants internalize weakly into COS and Chinese hamster ovary cells (18). Interestingly the pLANA8 mutant, which has a cytoplasmic tail of only nine amino acids, diffuses with a similar diffusion coefficient as the "wild-type" receptor, which has a complete cytoplasmic domain of 542 amino acids with intrinsic enzymatic activity and various regulatory sites. A similar diffusion coefficient was measured for the membrane IgG of lymphocytes ($D = 2 \times 10^{-10}$ cm²/s) (8), which possesses only three cytoplasmic amino acids. Furthermore, deletion of most of the 31 cytoplasmic residues of H-2L^d antigen did not affect either its lateral diffusion coefficient or the amount of mobile molecules in the plane of the membrane (9).

We have expressed these and other constructs in CHO cells (18), which are devoid of EGF receptors, and similar constructs in NIH-3T3 cells,² and studied their capacity to undergo receptor-mediated endocytosis. We have obtained several endocytosis-defective mutants that are mutated in the cytoplasmic domain of the receptor. These include the products of pLAB71 and pLANA8 constructs. However, other receptor constructs mutated in the cytoplasmic domain that

Table II. Diffusion Coefficients and Mobile Fractions of EGF Receptor Mutants

| Cell type and construct | No. of samples | Diffusion, cm ² /s | % Mobility |
|-------------------------|----------------|--------------------------------|-------------|
| pLSX construct in COS | 26 | $1.2 \pm 0.7 \times 10^{-10}$ | 61 \pm 21 |
| pLSXC construct in COS | 29 | $1.6 \pm 1.4 \times 10^{-10}$ | 59 \pm 21 |
| pLAB71 in COS | 23 | $1.5 \pm 0.77 \times 10^{-10}$ | 70 \pm 18 |
| pLANA8 in COS | 27 | $1.2 \pm 0.7 \times 10^{-10}$ | 77 \pm 21 |
| NBD-PE in COS | 37 | $4.4 \pm 2.1 \times 10^{-9}$ | 78 \pm 18 |
| Rh-EGF A-431 | 15 | $2.3 \pm 1.3 \times 10^{-10}$ | 49 \pm 19 |
| NBD-PE A-431 | 26 | $1.6 \pm 0.7 \times 10^{-9}$ | 84 \pm 12 |

All diffusion measurements were done at room temperature (beam radius, 0.699 μm). The spread in the diffusion constant could be circumvented by separation of each measurement into slow and fast diffusing populations. However, histograms of each of the diffusion coefficient of each mutant did not separate into two such populations nor were any trends found between the mobility of the constructs when an arbitrary 20% of the samples were chosen for the slow diffusion population. The diffusion coefficients and percent mobility values are represented as mean \pm SD. 250 ng/ml Rh-EGF added to cells for 1 h at 4°C. 20 μl /1,000 μl nitrobenzoxadiazole-phosphatidylethanolamine (NBD-PE) (gift from M. Shinitzky, Weizmann Institute) was added to cells for 1 h at 4°C. Beam radius for lipid probe measurements equals 1.54 μm .

2. Prywes, P., E. Livneh, A. Ullrich, and J. Schlessinger. Manuscript submitted for publication.

abolish the kinase activity can internalize, thus indicating that the kinase activity per se may not be required for endocytosis.² We are currently analyzing more constructs with mutations in the cytoplasmic region to better define the sequences that control the endocytosis of EGF receptor.

Clearly a deletion of 533 amino acid residues from the cytoplasmic domain of EGF receptor does not affect its lateral mobility. Hence, most of the cytoplasmic domain of EGF receptor may not interact with the underlying cytoskeletal network, and it does not restrict receptor diffusion by "steric hindrance." However, it is possible that the small cytoplasmic tail of nine amino acids that remains in the pLΔNA8 mutant can already interact with the underlying cytoskeleton. At least three additional mechanisms (9) may be relevant for the regulation of the lateral diffusion of the EGF receptor. (a) The concentration of the proteins in the plasma membrane is high, thus possibly providing an additional constraint on receptor mobility. (b) The EGF receptor may interact with another membrane protein that is associated with the cytoskeleton. (c) It is possible that the lateral mobility of EGF receptor is hindered by dominant interactions between the extracellular domain of the EGF receptor and the glycocalyx. These three hypotheses may also explain the constrained lateral diffusion ($D = 3 \times 10^{-10}$ cm²/s) of sterol dextran incorporated in the plasma membrane of animal cells in culture (28). Whatever the mechanism, it is clear that a deletion of most of the cytoplasmic domain of EGF receptor, which leads to the loss of several functional sites, is not required for the restriction of the lateral mobility of the EGF receptor kinase.

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