Overexpression of Epidermal Growth Factor Receptor in NIH-3T3-transfected Cells Slows Its Lateral Diffusion and Rate of Endocytosis

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Abstract. Interactions between membrane proteins are believed to be important for the induction of transmembrane signaling. Endocytosis is one of the responses which is regulated by both intracellular and extracellular signals. To study such interactions, we have measured the lateral mobility and rate of endocytosis of epidermal growth factor receptor in three transfected NIH-3T3 cell lines (HER84, HER22, and HER82) expressing 2×10^4, 2×10^5 and 1.5×10^6 EGF-receptors per cell, respectively. Using rhodamine-labeled EGF (Rh-EGF) and rhodamine-labeled monoclonal anti-EGF-receptor antibody (Rh-mAb-108), we measured twofold decreases in the lateral diffusion coefficients for each ~10-fold increase in EGF-receptor concentration. Since steric effects cannot account for such dependence, we propose that protein mobility within the membrane, which is determined by the rate of motion between immobile barriers, decreases due to aggregate formation. The rate of endocytosis also decreases twofold between the HER84 (2×10^4 receptors/cell) and HER22 (2×10^5 receptors/cell) cell lines, suggesting that it is diffusion limited. The comparable rates of endocytosis of the HER82 and HER22 cell lines suggest that at high receptor density endocytosis may be limited by the total number of sites for receptors in coated-pits and by their rate of recycling.

THE membrane receptor for epidermal growth factor (EGF)[1] is a glycoprotein of molecular weight of 170,000, which is composed of an extracellular EGF-binding domain, a 23 amino acid transmembrane stretch, and cytoplasmic domain containing a protein tyrosine kinase function and several autophosphorylation sites (reviewed in Schlessinger, 1986). EGF binding to its receptor induces several cellular processes such as an increase in Na^+/H^+ exchange (Moolenaar et al., 1983), calcium fluxes (Sawyer and Cohen, 1981) and increased phosphorylation of various substrates (reviewed in Hunter and Cooper, 1985). These early responses to EGF stimulation are probably responsible for initiating other events which eventually lead to DNA replication and cell proliferation. EGF binds to receptors which are initially diffuse on the plasma membrane, subsequently, trapped in clathrin-coated pits and then internalized (Schlessinger et al., 1978; Haigler et al., 1978, 1979). It is not yet understood how ligand binding to the receptor activates intracellular signals and endocytosis. A plausible mechanism for the activation of transmembrane signals is through lateral interactions between receptors, which can be examined by measuring receptor mobility. Lateral diffusion of membrane proteins in live cells is ~10-fold slower than the diffusion of lipids in the same cells. Diffusion of proteins in artificial lipid bilayers is comparable to lipid diffusion and agrees with the theoretical calculations for the diffusion of a cylinder embedded in a lipid bilayer (Saffman and Delbruck, 1975) neglecting the small hydrodynamic contribution of the portions extending out from the bilayer. Thus, the discrepancy observed between protein and lipid lateral diffusion coefficients must result from constraints on the protein motion.

Constrained membrane protein mobility can be a result of (*a*) aggregate formation, (*b*) steric interactions due to high concentrations of protein in the membrane or (*c*) interactions between these proteins and immobile elements in the cell interior or in extracellular matrix. Recently, we have shown that large deletions in the cytoplasmic portion of the EGF-receptor do not influence its lateral diffusion (Livneh et al., 1986) suggesting that the dominant factor constraining receptor mobility is not direct attachment to the cytoskeleton. Associations between major histocompatibility antigens and components of the extracellular matrix have been suggested as one of the sources of constrained lateral diffusion in

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1. *Abbreviations used in this paper*: EGF, epidermal growth factor; FPR, fluorescence photobleaching recovery; HDME, hepes-buffered (20 mM) DME containing 10% FCS and lacking phenyl red; NBD-PE, N-(4-nitrobenz-2-oxa-1,3-diazole), phosphatidylethanolamine; Rh-EGF, rhodamine-labeled epidermal growth factor; Rh-mAb-108, rhodamine-labeled monoclonal IgG_{2a} anti-EGF receptor antibody.

confluent cultured cells (Wier and Edidin, 1986). Slow receptor mobility could limit the internalization of EGF-receptor. Therefore, it is interesting to examine if changes in diffusion rates correlate with other cellular changes, such as, the rate of endocytosis.

We have selected various transfected NIH-3T3 cell lines shown to be devoid of endogenous murine EGF-receptor yet vary in the density of human EGF-receptor expressed on the cell surface. By using these cell lines, we can determine the relationship between the density of EGF-receptors in the membrane, receptor lateral mobility and the kinetics of receptor endocytosis. We show that for a 75-fold increase in the number of EGF-receptors per cell, there is a fourfold decrease in the lateral diffusion coefficient and a threefold decrease in the relative rate of EGF internalization. On the basis of these results, we propose that the rate of motion is reduced because of aggregate formation, and that the rate of ligand internalization may be limited by the total number of EGF receptors in coated pits and by their rate of recycling.

Materials and Methods

Cells

NIH-3T3 cells were transfected with a full-length cDNA construct of human EGF-receptor and three cell lines were selected and characterized for the expression of EGF-receptor (Prywes et al., 1986). These cell lines were grown at 37°C and 5% CO_2 in DME containing 10% FCS supplemented with 5 mM glutamine and combined antibiotics. For fluorescence photobleaching recovery (FPR) measurements (reviewed in Schlessinger and Elson, 1982), cells were grown on 18-mm^2 glass coverslips to 30% confluency in 35-mm tissue culture dishes. Cells were washed twice with 20 mM Hepes-buffered DME containing 10% FCS and lacking phenyl red (HDME). The coverslips were then inverted onto parafilm containing a drop (usually 4 μl) of either 395 μg/ml Rh-mAb-108 or 49 μg/ml Rh-EGF and incubated for 5 min at room temperature. Cells were washed twice and placed on a glass slide containing 6 μl of HDME. Cells were labeled with N-(4-nitrobenz-2-oxa-1,3-diazole)-phosphatidylethanolamine (NBD-PE) by adding 10 μl to a coverslip bathed in 1 ml of HDME and incubated for 20 min at 37°C.

Preparation of Rhodamine-labeled Protein Probes

Rh-EGF was prepared by mixing 100 μg epidermal growth factor (I.D.L., Jerusalem) dissolved in PBS with 25 μl of 1 M sodium carbonate buffer pH 9, with 75 μg of lysamine rhodamine B sulphonyl chloride (Chen, 1969) in three aliquots, 20 min apart with occasional vortexing. This reaction mixture was then loaded on a 1 × 10-cm Sephadex G-10 (Sigma Chemical Co., St. Louis, MO) column equilibrated and eluted with 10 mM sodium phosphate buffer, pH 8, to separate the dye bound to protein from the unbound dye. The labeling ratio of fluorophore to EGF for this procedure was generally 1:2. This is because at least half of EGF molecules have a blocked amino terminal residue. Monoclonal antibody-108 IgG_{2a} (Bellot, F., R. Kris, and J. Schlessinger, manuscript in preparation) was labeled with rhodamine by the procedure described above except that 30 μg of lysamine rhodamine B sulfonyl chloride per milligram of antibody were added in three aliquots and eluted on a 1 × 10-cm Sephadex G-25 column. This procedure yielded ~1–2 fluorophores per antibody molecule.

Preparation of NBD-PE

NBD-PE was prepared by the procedure of Vaz and Hallmann (1983), and kindly provided by Meir Shinitzki.

Fluorescence Photobleaching Recovery

Lateral diffusion of fluorescently labeled EGF-receptor was measured by the procedure described by Koppel et al., (1976). In brief, a 200–400 mW laser beam (488 nm for NBD-PE, 515 nm for Rh-EGF) is focused to a spot on the sample slide by a Ziess universal microscope using a 63 × N.A. 1.4 objective lens. The laser beam is stopped by a Uniblitz electronic shutter, and a monitoring beam is split away from that beam to bypass the shutter and is precisely recombined with it by four internal reflections using two rectangular glass blocks which also attenuate the beam by ~40,000-fold. A computer-controlled pulse opens the shutter for 40–250 ms and allows the unattenuated beam to bleach the fluorophore at the focused spot. The monitoring beam then measures the recovery of the fluorescence intensity at this same spot. The dynamics of fluorescence recovery are measured by a photon counting system consisting of a dry ice–cooled photomultiplier containing gallium-arsenate plates (RCA) supplied with 1,800 V which is gated during the bleach. The signal is pre-amplified and photon pulses are discriminated (Ortec 9301, Ortec 9302, Elscint ED-N-2, and Elscint TFA-N-B.) The final signal enters a microcomputer-based signal averager with a photon counting interface. Approximately 30% of the data obtained was a sum of two traces taken on the same spot in the cell. Data is analyzed by a VAX 11/780 computer by fitting with the theoretical rate of recovery after photobleaching (Axelrod et al., 1976; Petersen et al., 1986). Beam size was measured as described by Schneider and Webb (1981), using TV imaging with an RCA SIT camera situated at the back image plane of the microscope. The image of the laser beam is reflected from a mirror at the microscope object plane and digitized by a Gould deAnza FD5000 image processor. The best gaussian fit was obtained by using a nonlinear least squares fitting procedure, and the magnification of the beam was determined from the digitized image of a calibration slide. FPR measurements were accomplished with a 1.43- and 1.64-μm beam spot radius.

Fluorescence Intensity Measurements

Fluorescence intensity of the different cell lines was measured by using a Ziess axiomat microscope equipped with a 100 × 1.3 N.A. planapo objective and epiluminescence with a 100-W mercury lamp (Osram). Fluorescent light was collected with a Paltier cooled (Products for Research, Inc.), photomultiplier (EMI) through an 8-μm diameter pinhole. Photon pulses were amplified, discriminated (Ortec 9301, 9302, Elscint TFA-N-1) and counted (Fluke, 1953*a*). Fifteen to thirty readings of different cells were recorded for each concentration. Background readings were determined by measuring areas not containing cells. Fluorescence intensity from nonspecific binding was measured by incubating cells with a 50-fold excess of unlabeled ligand in addition to the labeled ligand.

Binding and Internalization of [^{125}I]EGF

NIH-3T3 cells expressing EGF-receptor, were seeded in 24-well Costar dishes at a density of 5×10^4 cells per well, 2 d before the binding experiments. Cells were washed twice with DME containing 0.1% BSA buffered with 20 mM Hepes, pH 7.5, and then incubated with various concentrations of [^{125}I]EGF in the same medium for 1.5 h at room temperature. Then the cells were washed twice with medium, and solubilized with 1 ml of 0.2 N NaOH. After 60 min at 37°C the radioactivity of the solubilized cells was measured. Nonspecific binding was determined by parallel incubations of each sample with 100-fold excess of EGF. Specific binding was calculated as total minus nonspecific cell associated radioactivity. Specific and nonspecific binding was determined in duplicates. [^{125}I]EGF was prepared by the lactoperoxidase method (Marchalonis, 1969). The number of receptors per cell and the dissociation constant (K_D) for their binding were determined from Scatchard plots. Data was analyzed using a computer program developed by Stephen Felder. The method of acetic acid cell treatment was used to measure the amount of internalized versus surface-bound [^{125}I]EGF (Haigler et al., 1980). Binding experiments were performed as described above except that binding was measured at various times at 37°C. [^{125}I]EGF (100 ng/ml) was added to cells in tissue culture dishes placed on ice. After different time periods at 37°C, the labeled cells were washed three times with PBS and treated with 0.5 N acetic acid containing 0.15 M NaCl for 6 min on ice. The acetic acid treated-cells, and the cells which were not exposed to this treatment were solubilized with 0.2 N NaOH and their radioactive content was determined. Specific and nonspecific bindings were determined in duplicates.

Results

To determine the number of receptors per cell for each cell line, quantitative binding studies were conducted with [^{125}I]EGF. The HER82 cell line has 1.5×10^6 receptors of which 18% are high affinity ($K_D = 1.62 \times 10^{-10}$ M) and

Table I. Binding Parameters for EGF Binding to EGF-Receptor in Transfected NIH-3T3 Cells

Cell type	K_D (High)	High affinity	K_D (Low)	Low affinity	Total number receptors
		%		%	
HER82	1.62×10^{-10}	18	6.02×10^{-9}	82	1.5×10^6
HER22	2.8×10^{-10}	15	4.7×10^{-9}	85	2.0×10^5
HER84	7.6×10^{-11}	20	2×10^{-9}	80	2.0×10^4

The binding parameters were determined from Scatchard plots as described in Materials and Methods.

82% low affinity ($K_D = 6.02 \times 10^{-9}$ M). The HER22 cell line contains 15% high affinity receptors and a total of 2×10^5 receptors per cell, and HER84 cell line contains ~20% high affinity receptors and a total of 2×10^4 receptors per cell (Table I).

Cells were labeled for 5 min with 200 ng of Rh-EGF. Short incubations with saturating concentration of rhodaminated EGF at room temperature have been used routinely for FPR measurements instead of the common long incubations with much lower concentrations at 4°C. Cold treatment arrests endocytosis, but may have slow recovering effects on internal cell structure such as microtubule reassembly (Gaskin et al., 1974). After the short incubation at room temperature, FPR experiments could be conducted with minimal cell trauma. However, part of the Rh-EGF molecules may at this time undergo endocytosis which will reduce the percentage of mobile surface receptors. Fluorescence measurements for the HER82 cell line were done to test binding at high concentration, short time labeling conditions. Saturation labeling of HER82 was achieved by incubation with 12 μl of 49 μg/ml Rh-EGF. A 4-μl drop saturated the fluorescence of the HER22 and HER84 cell lines. Similar results were obtained for Rh-mAb-108. An eightfold decrease in fluorescence intensities was observed between HER82 and HER22 cell lines in accordance with the amount of [^{125}I]EGF bound to these two cell lines. Initial HER84 cell fluorescence intensity could not be evaluated with sufficient precision due to background, but FPR experiments which record changes in intensity could be measured (see Fig. 1), but could not yield reliable data for the immobile fraction. Nonspecific binding using a 50-fold excess of unlabeled EGF or mAb-108 in addition to the fluorescent ligand accounted for ~80% of the fluorescence intensity in HER84 cells; yet FPR measurements of the cells under these conditions showed no recovery at minute-time-scales. Variations in the initial fluorescence intensities exist between cells on the same coverslip; however, the precision of the measurements on single cells did not make it possible to resolve a correlation between fluorescence intensities and the experimental recovery times.

The averaged lateral diffusion coefficient for the population of HER82 cells labeled with Rh-EGF was twofold slower than the diffusion coefficient measured for HER22 cells. A twofold increase in the diffusion coefficient was also observed between HER22 and HER84 cell lines (Table II). Lateral diffusion obtained for these three cell lines using a rhodaminated IgG_{2a} monoclonal antibody which recognizes specific epitopes on the extracellular domain of the EGF-receptor (Rh-mAb-108), were similar to the diffusion coefficients measured by Rh-EGF (Table II). In contrast, diffusion of the fluorescent lipid NBD-PE, was similar in all three cell lines, and is ~twofold faster than the fastest EGF-receptor diffusion coefficient measured in HER84 cells. Mobile fractions measured for each of the cell lines was unusually low; however, control measurements done on A431 cells under the same experimental conditions yielded mobile fractions consistent with those found in the literature of 40–70% for EGF-receptor, and 80 to 90% for NBD-PE. NIH-3T3 cells have high levels of internal fluorescence that may be due to flavins and may account for these results.

Internalization of [^{125}I]EGF was characterized for all three cell lines using the commonly applied acetic acid wash protocol (Haigler et al., 1980). Within 5 min HER82 cells internalize 2.9×10^5 EGF molecules per cell where 1.5×10^6 receptors are occupied initially. HER22 cells internalize 5.2×10^4 EGF molecules per cell, when 2×10^5 receptors are bound initially; and HER84 cells internalize 1.2×10^4 EGF molecules per cell within 5 min when 2×10^4 receptors are initially occupied. Although, the absolute number of internalized receptors increases with increasing number of receptors expressed on the cell surface, the relative rate of internalization decreases. Fig. 2 shows that within 5 min HER82 cells internalize 19% of its total; HER22 cells, 26% of its total and HER84 cells, 60% of its total amount of ligand occupied receptors per cell. All three cell lines had essentially finished internalizing [^{125}I]EGF after 1 h at 37°C al-

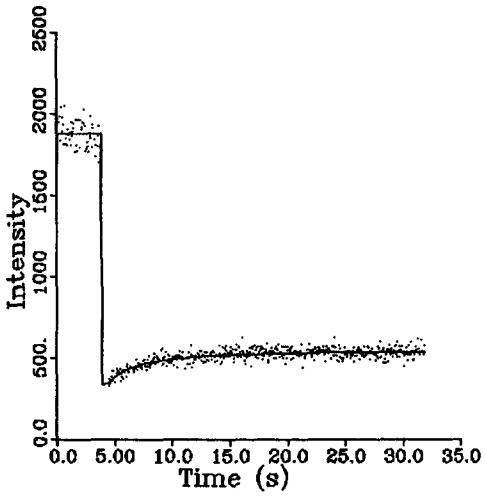

Figure 1. A typical FPR curve of HER84 cells labeled with 4 μl of 49 μg/ml of Rh-EGF for 5 min at room temperature as described in Materials and Methods. The data were analyzed by a least-square two parameter fitting program (Petersen et al., 1986) yielding the experimental recovery time (τ_D) and the mobile fraction (mf). $D = x^2/4\tau_D$. For this curve, $\tau_D = 1.51$ s, mf = 13.6% and the beam radius x = 1.64 μm.

Table II. Diffusion Coefficients and Mobile Fractions for EGF Receptor in Transfected NIH-3T3 Cells

Probe (no. of samples)	No. of receptors Cell line	D_T(cm²/s) (SD‡)	Mobile (SD‡)
			%
Rh-EGF (49)	1,500,000 (HER82)	6.6×10^{-10} (4.4)	27 (15)
Rh-Ab-108 (61)	1,500,000 (HER82)	6.0×10^{-10} (2.8)	22 (8)
NBD-PE (28)	(HER82)	49×10^{-10} (24)	17 (13)
Rh-EGF (49)	200,000 (HER22)	15×10^{-10} (7.0)	22 (14)
Rh-Ab-108 (29)	200,000 (HER22)	11×10^{-10} (8.0)	18 (6)
NBD-PE (24)	(HER22)	35×10^{-10} (21)	17 (11)
Rh-EGF (25)	20,000 (HER84)	28×10^{-10} (16)	21 (10)
Rh-Ab-108 (33)	20,000 (HER84)	20×10^{-10} (14)	16 (6)
NBD-PE (30)	(HER84)	49×10^{-10} (22)	23 (5)

‡ Standard Deviation

though in the cases of HER22 and HER82 cells not all of the ligand was internalized.

Discussion

In this paper, we have shown that an increase in the density of receptors expressed on the cell surface yields a decrease in the average lateral diffusion coefficient and the rate of ligand internalization when a constant concentration of Rh-EGF or Rh-mAb-108 was applied.

The dependence of the lateral diffusion coefficient on the density of protein in the membrane has been modeled by hard hexagon Monte Carlo simulations (Pink, 1985). In this simulation, linear increases in fractional occupancy yielded almost linear decreases in the lateral diffusion coefficient. We are unable to determine experimentally the fractional occupancy in our preparations since we cannot determine the total content and area covered by all the proteins in the membrane. Still, our data show that each 10-fold decrease in the number of EGF-receptors yields a twofold increase in lateral mobility. These results for cells expressing differing amounts of EGF-receptor definitely diverge from linearity, indicating that simple steric collisions between mobile membrane proteins cannot account for the change in lateral mobility. Therefore, we conclude that there is an increase of other types of interactions with increasing concentrations of EGF-receptor.

Increasing the amount of receptor in the membrane may drive the receptor to aggregate. Measurements of the rotational diffusion coefficient of labeled EGF bound to A431 cells (Zidovetzki et al., 1981) or to membrane preparations (Zidovetzki et al., 1986), suggests the formation of EGF-induced receptor aggregates. Sucrose gradient centrifugation (Biswas et al., 1985) and nondenaturing PAGE (Yarden and Schlessinger, 1987) have also shown the presence of EGF-receptor oligomers. The Saffmann and Delbruck (1975) model for diffusion within a membrane, predicts a logarithmic dependence on radius yielding approximately a twofold decrease in diffusion for a 50-molecule aggregate in comparison with the monomer. However, if the dependence of the lateral diffusion was only due to aggregate size, then an additional twofold decrease in the diffusion coefficient would indicate an approximate change from 50 to 2,500 molecules per aggregate. An aggregate of 2,500 molecules can be seen as a fluorescent patch; yet, we observe that during our measurements, labeling with Rh-EGF on the cell surface appears diffuse. The fluorescent patches which develop later corresponds to internalized vesicles containing Rh-EGF-receptor complexes (Hillman and Schlessinger, 1982).

Immobile elements within the membrane may act as a labyrinth of barriers through which the mobile proteins diffuse. Percolation theories describe the dependence of diffusion on the probability of crossing the barriers in such a labyrinth (Saxton, 1982). The crossing probability is determined by the ratio between diffuser diameters and the openings between barriers. A twofold decrease in receptor diffusion coefficient could result from increasing the average aggregate size which decreases the number of receptors that can freely

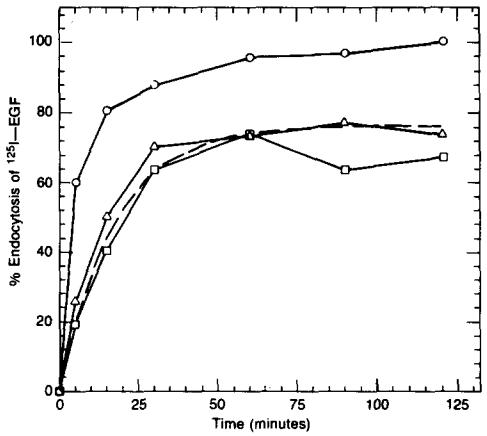

Figure 2. Relative internalization of [^{125}I]EGF was determined by taking the ratio of cpm resistant to the acetic acid wash to total cpm determined by binding [^{125}I]EGF at 4°C (see Materials and Methods) (□) represents values obtained for HER82; (△) for HER22; and (○) for HER84 cell lines, respectively. The total number of internalized receptors is simulated by assuming partial recovery of recycled coated-pits. Taking $e_o = 2.9 \times 10^5$ sites initially occupied by receptors and internalized, and $e_1 = qe_o$ sites recycled and restored after five minutes, we get for the total number of internalized receptors after T minutes (or $T/5$ cycles):

$$\sum_{j=0}^{T/5} ej\, q^j = e_o\,\frac{(1 - q^{T/5+1})}{(1 - q)}.$$

Dashed lines represent the simulation plotted for q = 0.75 which highly correlates to the measurements for HER82 cells containing 1.5×10^6 receptors initially.

diffuse. The average aggregate size is most sensitive to changes at concentrations approximately equivalent to the inverse of the aggregation constant ($1/K_{agg}$; van Holde, 1985). Purified EGF-receptor preparations of A431 cells, which contain $2–4 \times 10^6$ receptors per cell, were found to contain 70–80% of the receptors in the dimer form in the presence of EGF (Yarden and Schlessinger, 1987), suggesting that the aggregation constant corresponds to lower concentrations of the receptor. If $1/K_{agg}$ is in the range of concentrations corresponding to 10^5 receptors per cell,[2] then in the HER82 line the receptor would be mostly aggregated; while in the HER84 line most receptors would be in monomeric form. Thus, it is possible to explain our results by assuming that the free paths between barriers are sufficiently large for unperturbed passage of monomers, but inhibiting for aggregates. If aggregates are totally immobile, our diffusion measurements could reflect the average lifetime of free monomers, which is dependent on the aggregate dissociation constant (K_{agg}) and the total receptor concentration.

It has been shown under equilibrium-binding conditions, that A431 cells which were treated with a high concentration of EGF (1.44 μg/ml) to bind the low affinity sites, had a diffusion coefficient of 2.6×10^{-10} cm^2/s (Rees et al., 1985).

2. To avoid the problems of defining concentration units in two dimensions, we do not define the value of K_{agg}.

At very low EGF concentration, there was no recovery of fluorescence suggesting that high affinity EGF-receptors are immobile. Our Scatchard analysis of EGF concentration down to 0.96 ng/ml indicates the presence of low and high affinity receptors in the HER82 cell line. However, our fast-labeling procedure for FPR measurements using a small drop of highly concentrated Rh-EGF does not allow for sufficient equilibration within the 5-min incubation period, and therefore, cannot select high from low affinity binding sites.

The relationship between the rate of lateral diffusion and the kinetics of endocytosis is not simple, since it involves several unknown parameters that describe the gathering, occupancy and internalization of EGF-receptor in coated pits. Assuming that the number of coated pits and the dynamics of internalization is similar for all three cell lines, we would expect that if 1.2×10^4 receptors were internalized in the HER84 cell line within 5 min, then 1.2×10^5 and 9×10^5 receptors per cell should be internalized for the HER22 and HER82 cell lines, respectively. Since we measure only 2.9×10^5 receptors for HER82, we can deduce that the total number of sites in coated pits during the first 5 min of internalization is saturated at about this level. Thus, coated pits for the HER22 and the HER84 cell lines would be 18% and 5% occupied, respectively. If entrance of coated pits into the cell were the rate-limiting step, then 2.9×10^5 coated-pit sites would provide for 100% internalization of EGF in both HER22 and HER84 cell lines within the first 5 min at 37°C. Since this is not the case, either diffusion to the coated pits or the frequency at which receptors are trapped is rate-limiting.

Keizer et al. (1985), using parameters obtained from low density lipoprotein which may also be applicable here, calculated that internalization of coated pits may partially depend on the diffusion of the low density lipoprotein receptor to coated pits. Our results support this calculation since there is a twofold decrease in both diffusion and endocytosis rates between HER84 and HER22.

In the case of the HER82 cell line, the high concentration of receptors eliminates the possibility that diffusion of receptors to coated pits and their trapping is rate-limiting since the coated pits are filled with receptors before internalization can take place. Thus, the relative rates of endocytosis of HER22 and HER82 is similar even though there is a twofold difference in their lateral diffusion coefficient.

Internalization of [^{125}I]EGF in the HER82 and HER22 cell lines does not reach 100% of the total receptor number found initially on the cell surface. After a 2-h incubation of the HER82 cell line with [^{125}I]EGF at 37°C, there is still a considerable number of EGF-receptors that should be available on the cell surface for internalization. If recycling of EGF-receptors would continue at a constant rate (Honegger et al., 1987), we would expect that the rate of endocytosis should remain constant even after 2 h. Since internalization of [^{125}I]EGF in all three cell lines reaches its final value after approximately 1 h, the factor limiting the rate of internalization may be common to all three cell lines. It can be postulated that only a certain percentage of the sites in coated pits are recovered when recycled back to the membrane. We can simulate the rate of endocytosis based solely on this percentage of recovery. Fig. 2 (*dashed line*) shows that for a 75% recovery per 5-min cycle period (which is roughly the time

it takes to detect internalized EGF), internalization reaches saturation after 1 h. Furthermore, these considerations closely correlate to the results obtained for HER82 cells where internalization is not diffusion-limited, and coated pits internalize fully loaded and at their maximal rate. In the diffusion limited cases of HER84 and HER22, the initial rates and total amounts of endocytosed molecules differ; yet, the total amount of internalization reaches its maximum within 1 h at 37°C.

Alternatively, our results could imply that large aggregates, which are practically immobile, cannot be trapped in coated pits. The normalized concentration of monomers as a function of total concentration, is sigmoidal. Thus, if we assume that $1/K_{agg}$ corresponds approximately to 10^5 receptors per cell, then at 2×10^4, 2×10^5, and 1.5×10^6 total receptor concentration, there would be ~90% of 2×10^4, 50% of 2×10^5 and 10% of 1.5×10^6 free monomers present, respectively (see Feher and Kam, 1985). The fivefold difference in free monomer concentration at any moment between HER84 and HER22 cell lines, and the less than twofold difference seen between HER22 and HER82 cell lines, correlates directly with the differences in rates of endocytosis reported.

In these estimations, we have assumed that the total number of coated pits initially on the membrane and their rate of internalization are similar for all three cell lines. Yet, we can think of another possibility, where receptor internalization could require the filling of coated pits before internalization. In this case, they would enter the cell faster at higher concentration of EGF. The fact that only 80% of the EGF was internalized in HER22 cells, although, there are initially enough sites in coated pits for all the receptors is not compatible with the mechanism for exclusive internalization of fully occupied coated pits. Yet, to examine this possibility directly, we address this problem in a separate study.

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