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Rotational Mobility of High-Affinity Epidermal Growth Factor Receptors on the Surface of Living A431 Cells†

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ABSTRACT: The rotational diffusion of epidermal growth factor (EGF) bound to its specific receptor on the surface of human carcinoma A431 cells was studied by means of time-resolved phosphorescence anisotropy measurements. The rotational mobility was measured on the total population of EGF receptors by using a saturating concentration of EGF conjugated with a phosphorescent label, erythrosin, or on the subpopulation of high-affinity EGF receptors by using a low concentration of labeled EGF. At 4 °C, the rotational correlation times for both the high-affinity and total (mostly low affinity) receptor populations were in the range of 60–100 μs. Elevation of the temperature to 37 °C resulted in a lengthening of the rotational correlation time of the total receptor population to 200–300 μs, confirming a previous study of receptor microaggregation. The high-affinity EGF receptors were completely immobilized at 37 °C (rotational correlation time >500 μs). The data are consistent with a model involving association of the cytoskeleton with the high-affinity receptors at 37 °C, but not at 4 °C.

Epidermal growth factor (EGF)[1] is a 6045-Da polypeptide which binds to specific membrane receptors (EGFR) present as integral membrane proteins in a wide variety of cells (Carpenter & Cohen, 1979; Adamson & Rees, 1981). Binding initiates a number of biochemical processes including activation of intrinsic tyrosine kinase activity (Cohen et al., 1982; Buhrow et al., 1983), increased ion and metabolite transport, and stimulated proliferation of the target cells (Gospodarowicz et al., 1978; Carpenter & Cohen, 1979). EGFR is one of the most studied cell-surface receptors. Its primary amino acid sequence has been deduced by Ullrich et al. (1984), who also provided a tentative identification of an external hormone binding domain, a single membrane-spanning domain, and a cytoplasmic domain which exhibits the tyrosine kinase activity. EGFR is a member of a family of receptors with certain common features in their mechanism of action [see Gill et al. (1987), Carpenter (1987), Yarden and Ullrich (1988), and Schlessinger (1988) for reviews].

Two general mechanisms have been suggested to explain the transmembrane signal transduction by EGFR (Schlessinger, 1986; Basu et al., 1986): (1) EGF binding induces a conformational change of the receptor that propagates through the membrane by a single membrane-spanning domain of EGFR (intramolecular mechanism); (2) EGF binding induces a change in receptor–receptor interaction leading to activation of the cytoplasmic tyrosine kinase (intermolecular mechanism). Both mechanisms have experimental support (Basu et al., 1986; Koland & Cerione, 1988; Yarden & Schlessinger, 1987a,b; Boni-Schnetzler & Pilch, 1987).

The intermolecular mechanism was proposed to involve "microclustering" of EGFR as a necessary prerequisite for the biological response (Schlessinger et al., 1983). Indeed, EGF induces rapid formation of small receptor aggregates (not distinguishable by light microscope) immediately following binding to EGFR (Carpenter & Cohen, 1976; Zidovetzki et al., 1981). Receptor aggregation was recently shown to be linked to a modification of the tyrosine kinase activity of EGFR (Carraway et al., 1989). Formation of microclusters of EGFR upon EGF binding was also demonstrated on cell-free A431 cell membrane suspensions (Zidovetzki et al., 1986; Azevedo & Johnson, 1991). Moreover, EGF-induced dimerization of EGFR has been observed with detergent-solubilized preparations of EGFR (Boni-Schentzler & Pilch, 1987; Yarden & Schlessinger, 1987a,b; Cochet et al., 1988).

A transition from a mobile to an aggregated state of a receptor takes place in other systems. For example, receptor immobilization was reported after binding of nerve growth factor to its cell-surface receptor (Levi et al., 1980). In the case of insulin, the biological effects of the hormone were mimicked by cross-linking the insulin receptor with specific anti-receptor antibodies [Kahn et al., 1978; Jacobs et al., 1978; see Gill et al. (1987) for a critique of the clustering hypothesis].

The change in mobility, however, does not have to involve receptor self-aggregation; association with other plasma membrane molecules or cytoskeleton would also result in reduced receptor mobility. In the latter case, complete immobilization of the receptors would be expected, which would

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[1] Abbreviations: EGF, epidermal growth factor; Er-EGF, EGF labeled with erythrosin 5'-isothiocyanate; [^{125}I]EGF, EGF labeled with ^{125}I; EGFR, EGF receptor(s); HA-EGFR, high-affinity EGFR; LA-EGFR, low-affinity EGFR; PBS, phosphate-buffered saline.

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allow the potential of distinguishing between the processes of receptor association with other mobile membrane proteins (including self-association) with possible residual, albeit reduced, mobility, from a process of cytoskeleton association resulting in total receptor immobilization. Such reduced, but measurable EGFR mobility was reported for EGFR at 37 °C, indicating the absence of a strong cytoskeleton interaction for most EGFR (Zidovetzki et al., 1981).

It has been found since that A431 and many other cell types possess two types of EGF receptors that differ with respect to their affinity for EGF: high-affinity EGF receptors (HA-EGFR) and low-affinity EGF receptors (LA-EGFR). The two receptor populations were demonstrated on a number of cells of different origin and cell lines. Thus, HA-EGFR were detected on A431 cells (2–12% of the total number of receptors) (Friedman et al., 1984; Rees et al., 1984; Defize et al., 1988), Swiss 3T3 cells (43%) (Collins et al., 1983), Balb/c 3T3 cells (3%) (Walker & Burgess, 1988), RAT-1 cells (25%) (Magun et al., 1980), human amniotic WISH cells (30%) (Karasaki et al., 1989), fetal rat lung cells (16%) (Oberg et al., 1988), and mouse mammary gland cells (37%) (Komura et al., 1986). Thus, high-affinity sites represent 1–43% of the total receptor population, and a maximum mitogenic effect of various growth factors is realized when a similar proportion of receptors is occupied. This finding has led to a hypothesis that the low-affinity receptors do not directly participate in the mitogenic response (Rees et al., 1984). Indeed, A431 cells are inhibited by high EGF concentrations, but stimulated at low ones (Kawamoto et al., 1983).

Rees et al. (1984) observed that HA-EGFR are laterally immobile at all temperatures in the range of 3–37 °C and suggested that this immobilization may be a consequence of association of some EGF receptors with immobile or slowly diffusing effectors. Subsequent studies reported that HA-EGFR on A431 cells interact with the cytoskeleton (Landreth et al., 1985; Wiegant et al., 1986).

In our previous publications (Zidovetzki et al., 1981, 1986), we characterized the rotational mobility of the total population of EGFR on A431 cells and cell-free membrane suspensions. Here, we extend these studies to provide a comparative description of the rotational mobilities of the total receptor population and the subpopulation of high-affinity binding sites. The results indicate a total immobilization of HA-EGFR at 37 °C, but not at 4 °C, and are consistent with association of HA-EGFR with the cytoskeleton at elevated temperature.

Materials and Methods

Reagents. EGF was purified from adult mouse submaxillary glands (Pelfreez Biological, Rogers, AR) as described by Savage and Cohen (1972) and quantified by assuming a molar extinction coefficient of 18 700 cm M^{-1} at 280 nm. ^{125}I-Labeled EGF ([^{125}I]EGF) was prepared according to Carpenter and Cohen (1976). EGF labeled at the α-amino group with erythrosin isothiocyanate (Molecular Probes, Inc., Eugene, OR) was prepared and purified by gel filtration and high-pressure liquid chromatography as described elsewhere (Azevedo & Johnson, 1991). The ability of Er-EGF to inhibit [^{125}I]EGF binding to A431 cells was similar to that of native EGF. The phosphorescence measurements were done in phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 7.9 mM Na_2PO_4, 1.5 mM KH_2PO_4, 0.87 mM $CaCl_2$, and 0.5 mM $MgCl_2$, pH 7.2. Cell culture reagents were obtained from Cellgro (Herndon, VA) and Gibco (Grand Island, NY).

Cells and Culture. A431-29i cells (Santon et al., 1986), provided by G. N. Gill, were grown in DMEM/F12 (1:1) medium supplemented with 10% fetal bovine serum, 29 mM $NaHCO_3$, 20 mM Hepes, 1×10^5 units/L penicillin G, and 100 mg/L streptomycin sulfate.

[^{125}I]EGF Binding Assays. The A431 cells in 24-well plates (Costar, Cambridge, MA) [$(1–5) \times 10^5$ cells/well] were kept in medium without bovine calf serum for 6–24 h prior to the measurements, then washed with PBS, and fixed with 2% formaldehyde for 30 min, which was then replaced by PBS. Such formaldehyde fixation was shown not to affect the number and the affinities of EGFR on A431 cells (Van Maurik et al., 1985; Torris et al., 1988). The assays were performed at room temperature, with [^{125}I]EGF concentrations ranging from 16 pM to 16 nM in an incubation volume of 100 μL. The nonspecific binding was measured under identical conditions, but in the presence of 3.3 μM native EGF. The analysis of the binding data was done with the LIGAND program (Munson & Rodbard, 1980; Munson, 1983).

Sample Preparation for Phosphorescence Measurements. Confluent cultures [$(3–6) \times 10^6$ cells per 9-cm plate] were used for labeling with Er-EGF. Cells were released from plates by 10-min incubation with 0.8 mM EDTA and then resuspended in PBS. Labeling with Er-EGF was performed at 4 or 37 °C for 1 h. Er-EGF was added to a final concentration of 100–150 nM for measurements of the total receptor population or 3 nM for low saturation level (HA-EGFR) measurements. The cells were then washed twice with PBS at the corresponding temperature and resuspended with 0.5 mL of PBS into a spectrophotometric cuvette. The suspension was deoxygenated by addition of glucose (to 50 mM) and glucose oxidase (to 25 μg/mL final concentration) (Englander et al., 1987).

Measurements of Rotational Diffusion by Time-Resolved Phosphorescence Anisotropy. The delayed luminescence spectrometer has been described elsewhere (Matayoshi et al., 1983; Jovin & Vaz, 1989). The rotational mobility of Er-EGF–receptor complexes was determined as in our previous publications (Zidovetzki et al., 1981, 1986). Samples containing $(6–8) \times 10^6$ cells/mL were excited at 514 nm by an excimer–dye laser combination, and emission was collected above 645 nm by using a combination of a 50% w/v $Na_2Cr_2O_2$ liquid filter (Parker, 1968) and a RG645 cutoff filter (Schott, Mainz, FRG). Individual records were accumulated at 10 Hz and averaged; a file consisted of 1000 decay records of the parallel and perpendicular polarized emission components. Usually, three to five files were averaged for analysis. Blank corrections were made by subtracting files generated under identical conditions but using cells processed without Er-EGF or in the presence of excess of unlabeled EGF. The total phosphorescence emission, $S(t)$, was calculated as $S(t) = I_\parallel + 2I_\perp$ where $I_\parallel$ and $I_\perp$ are the parallel and perpendicular emission components, respectively. The data were fit to a two-component exponential decay function using a Marquardt nonlinear least-squares algorithm. The emission anisotropy, $r(t)$, was calculated as $r(t) = (I_\parallel - I_\perp)/S(t)$. In most cases, the data were adequately described by a weighted fit of $r(t)$ to a monoexponential decay function: $r(t) = \alpha e^{-t/\phi} + r_\infty$, where ϕ, the rotational correlation time, is an inverse function of the rotational diffusion constant and r_∞, the limiting anisotropy, reflects the anisotropic character of the rotational mobility of EGFR in the plasma membrane. A more complete description of the formalism used can be found in Cherry (1978), Zidovetzki et al. (1981), and Jovin and Vaz (1989).

Results and Discussion

A Scatchard-type plot of the binding of [^{125}I]EGF to fixed A431 cells is shown in Figure 1. Marquardt fits to a two-site

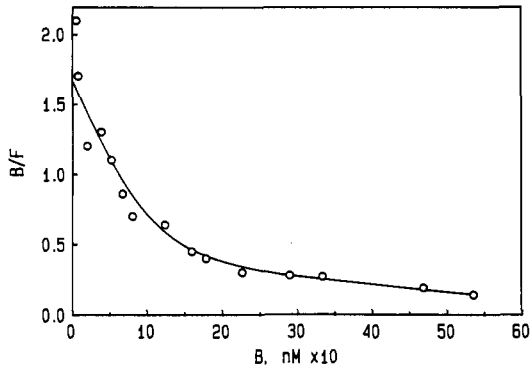

FIGURE 1: Scatchard analysis of [^{125}I]EGF binding to fixed A431 cells (see Materials and Methods). The line is drawn by using the best-fit parameters obtained from the LIGAND program: K_1 = 1.4 × 10^9 M^{-1}; K_2 = 2.8 × 10^7 M^{-1}; 1.8 × 10^6 receptors/cell; 12% HA-EGFR. Data points represent the mean of duplicate samples with nonspecific binding subtracted.

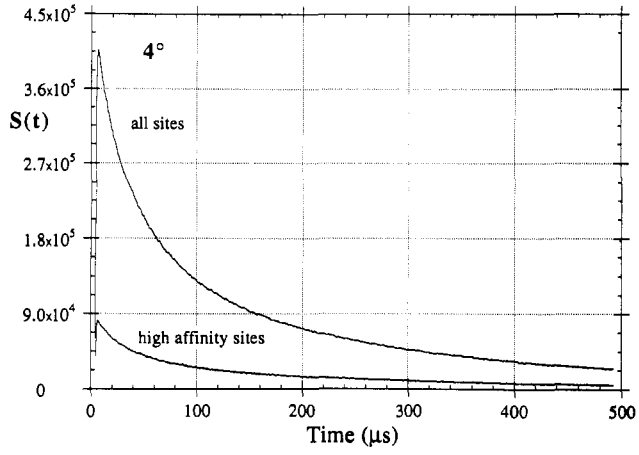

FIGURE 2: Decays of phosphorescence emission of Er-EGF-labeled A431 cells at a saturating concentration of Er-EGF ("all sites") or a low concentration of Er-EGF ("high affinity sites"). The y axis is in accumulated digital units; each trace is digitized with 8-bit resolution. The apparent increase of intensities in the beginning of the curves is due to gate-on function of the photomultiplier and was excluded from the data analysis. The data were digitized at 0.5 μs/channel.

model consistently gave significantly better results than analysis according to a one-site model. The total number of receptors per cell was in the range 1.2 × 10^6–1.8 × 10^6, less than that reported by Santon et al. (1986), but similar to values from other studies on the A431 cell line (Fabricant et al., 1977; Gamou et al., 1984). The percentage of HA-EGFR obtained in different assays varied considerably in the range of 12–35%. Such a variation has been pointed out by Kermode and Becker (1986) and is common for the two-site fits. The nonspecific binding was generally 3–6% of the total bound [^{125}I]EGF. The cells were typically incubated with 3 nM Er-EGF for measurements of HA-EGFR or with 110 nM Er-EGF for measurements of total sites. After corresponding incubations and washings, the phosphorescence intensities in HA-EGFR measurements were 20–25% of those with the total receptor population (Figure 2), consistent with the data obtained from [^{125}I]EGF binding assays and Scatchard analyses.

The phosphorescence decay curves were fit to biexponential functions (Figure 2). No statistically significant differences were found between the lifetimes and relative amplitudes of total and high-affinity receptor-bound Er-EGF (Table I), indicating that the chromophore environment of the two receptor populations was similar.

Table I: Phosphorescence Lifetimes of Er-EGF Bound to A431 Cells

receptor population	temp (°C)	τ_1 (μs)	τ_2 (μs)	rel amplitudes (%) amp 1	amp 2
total[a]	4	32 ± 4	164 ± 12	55 ± 5	45 ± 5
high affinity[b]	4	32 ± 4	160 ± 11	54 ± 4	46 ± 4
total[a]	37	21 ± 4	148 ± 20	57 ± 8	43 ± 8
high affinity[b]	37	20 ± 3	134 ± 18	66 ± 8	34 ± 8

[a] Labeled with a saturating concentration of Er-EGF. [b] Labeled with a low concentration of Er-EGF. The errors are given as standard deviations.

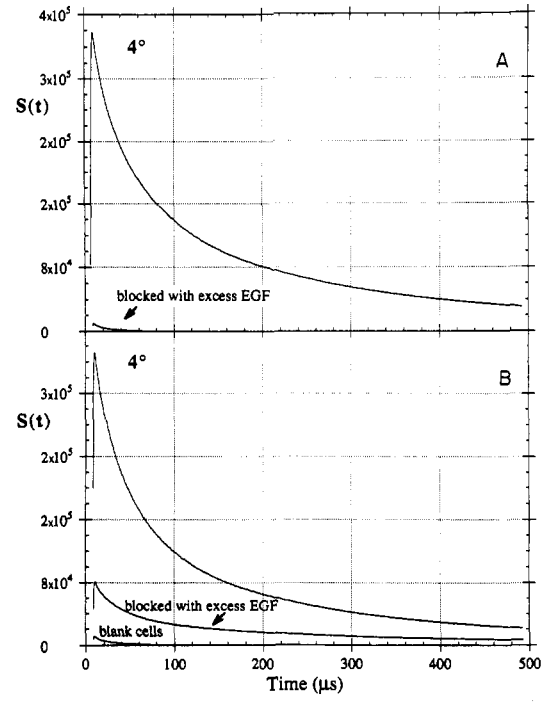

FIGURE 3: Phosphorescence emission decays of Er-EGF-labeled A431 cells in the absence or presence of a large excess of native EGF (3 μM). (A) Saturating concentration of Er-EGF. (B) Low concentration of Er-EGF (high-affinity site labeling). The emission from unlabeled A431 cells is included for comparison.

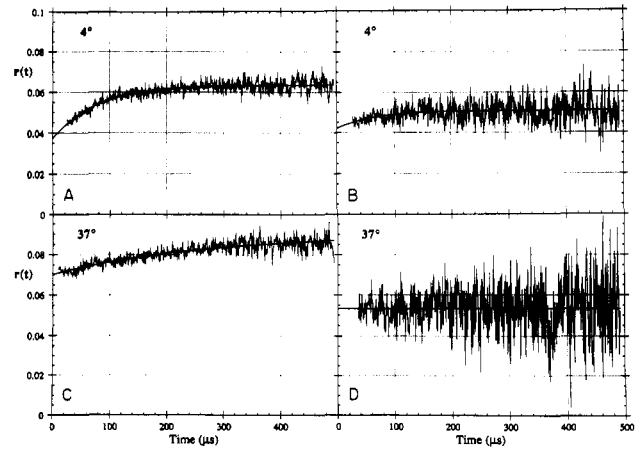

FIGURE 4: Anisotropy decay curves of Er-EGF–receptor complexes. The smooth lines were generated with the best-fit parameters given in Table II. (A, C) Incubated with saturating concentrations of Er-EGF; (B, D) incubated with low concentration of Er-EGF (high-affinity site labeling).

The specificity of Er-EGF binding to the receptors was verified by incubating the cells with Er-EGF in the presence of a large excess of unlabeled EGF (Figure 3). The phosphorescence intensity contributed by nonspecifically bound EGF constituted less than 3% of the total signal in the case

Table II: Phosphorescence Anisotropy Decay Parameters of Er-EGF Bound to A431 Cells

receptor population	temp (°C)	ϕ (μs)	α	r_∞
total[a]	4	70–100	−0.026 ± 0.004	0.07 ± 0.01
high affinity[b]	4	60–80	−0.012 ± 0.004	0.05 ± 0.01
total[a]	37	200–300	−0.024 ± 0.004	0.09 ± 0.01
high affinity[b]	37	∞		0.06 ± 0.01

[a] Labeled with a saturating concentration of Er-EGF. [b] Labeled with a low concentration of Er-EGF.

of the total sites (Figure 3A) and ~15% of the signal in the case of the HA-EGFR (Figure 3B).

The anisotropy decay curves of the total and high-affinity receptor populations at 4 and 37 °C are given in Figure 4, and the results of the analyses are summarized in Table II. The rotational correlation times of the total receptor population at 4 °C ranged between 70 and 100 μs (Table II), in good agreement with our previous work (Zidovetzki et al., 1981). It is now generally accepted that the receptor spans the membrane only once, with most of the receptor mass being outside of the plasma membrane (Ullrich et al., 1984). The value of the rotational correlation time obtained at 4 °C (70–100 μs) would thus probably correspond to a small cluster, rather than to a single receptor molecule, a possibility that was discussed in our original publication.

At low concentrations of Er-EGF, when mainly HA-EGFR are occupied (Figure 4B,D), the results are different. The rotational correlation time of HA-EGFR at 4 °C (60–80 μs) was similar to that of the total receptor population; however, the amplitude of the anisotropy decay was significantly smaller (Table II). The lower amplitude and residual anisotropy of low saturation experiments cannot be explained by an increased amount of free Er-EGF dissociated from HA-EGFR, because, in both cases (i.e., measurements of total receptor and HA-EGFR), the signals in the supernatant following centrifugation were ~20% of the total. The lower amplitude of the HA-EGFR anisotropy decay at 4 °C may indicate that a process of fast probe depolarization on a time scale faster than 1 μs is more prominent than in the case of the total receptor population. An alternative explanation is that there is a difference in the orientation of the erythrosin chromophore relative to the plane of the membrane when the Er-EGF is complexed to HA-EGFR or the total (mostly LA-EGFR) receptor population.

A dramatic difference between the total receptor population and the high-affinity sites was observed at 37 °C (Figure 4C,D). The total EGFR still exhibited a discernible mobility, albeit slower than at 4 °C. The rotational correlation times increased at the higher temperature to ~200–300 μs, reflecting microaggregation of the Er-EGF-bound receptors. The concomitant increase in the residual anisotropy from 0.07 to 0.09 is also consistent with the formation of EGFR aggregates because microaggregation would increase the orientational constraints (Kinosita et al., 1984) of the Er-EGF–EGFR complexes.

In contrast, on the time scale of the phosphorescence experiment, the HA-EGFR were completely immobilized when incubated with Er-EGF at 37 °C and observed within a short time (30–40 min) after the start of the incubation with Er-EGF (Figure 4D). Longer incubations at 37 °C did not produce reproducible pattern of the phosphorescence anisotropy decays; this phenomenon deserves further investigation.

In comparing the present results with the lateral mobility measurements on the same system, it is notable that at 4 °C the HA-EGFR are rotationally mobile, but laterally immobile (Rees et al., 1984). It has been noted before that discrepancies between rotational and lateral diffusion measurements could be due to the long range of the latter (the measurements detect protein mobility over a distance of ≥1 μm), which can thus be a function of interactions other than the viscous drag of the lipid membranes, for example, interactions with the underlying cytoskeletal network or the extracellular matrix (Schlessinger, 1983). However, we observed a complete immobilization of HA-EGFR at 37 °C on a time scale of ≤500 μs (Figure 4D). The observed immobilization may be due to formation of large aggregates of HA-EGFR. Alternatively, the immobilization may be caused by a strong interaction of HA-EGFR with the cytoskeleton. Indeed, cytoskeleton association of HA-EGFR on A431 cells was reported by Landreth et al. (1985), Wiegant et al. (1986), Roy et al. (1989), van Bergen en Henegouwen et al. (1989), and Lichtner and Schirrmacher (1990). Cytoskeleton connection was also reported for other cell membrane receptors, and its possible role in transmembrane signal transduction was discussed by van Bergen en Henegouwen et al. (1989) and Lichtner and Schirrmacher (1990). The general topic of membrane–cytoskeleton interaction, including receptor–cytoskeleton interaction, was the subject for a review by Geiger (1983).

We observed consistent immobilization of HA-EGFR only if the cells were exposed to EGF at 37 °C, pointing out a potentially complex dependency of the HA-EGFR–cytoskeleton association on the thermal and temporal sequences defining the conditions of EGF treatment. Further experiments at the cellular level are required to elucidate the details of this process.

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