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Lateral Diffusion of Epidermal Growth Factor Complexed to Its Surface Receptors Does Not Account for the Thermal Sensitivity of Patch Formation and Endocytosis†

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ABSTRACT: The patching and endocytosis of EGF (epidermal growth factor) bound to A-431 cells (a human epidermoid carcinoma line) are temperature-sensitive processes which are completely inhibited at 4 °C. Receptor-mediated endocytosis generally occurs through coated regions, and EGF bound to its membrane receptor must diffuse laterally to these points of internalization. In this work we investigated the thermal sensitivity of the lateral diffusion of EGF receptor complexes and the thermal sensitivity of the patching and endocytosis of the hormone receptor complexes. Using the fluorescence photobleach recovery technique, we measured the lateral diffusion coefficients of a fluorescent derivative of EGF as a function of temperature. The lateral diffusion coefficient (D) increased gradually from 2.8×10^{-10} cm^2/s at 5 °C to 8.5×10^{-10} cm^2/s at 37 °C, and no phase transition was detected. Neither was a phase transition detected when we measured the diffusion coefficient of fluorescent lipid probes over this temperature range. From a calculation of the collision frequency of the occupied EGF receptors with coated regions using our measured values of D at 5 and 37 °C, we conclude that diffusion is not the rate-limiting step for either endocytosis or patching.

Epidermal growth factor (EGF)[1] is a 6045-dalton polypeptide which binds to specific membrane receptors on various epidermal, epithelial, and fibroblastic cells (Carpenter & Cohen, 1979). EGF initiates rapid responses such as the uptake of metabolites, phosphorylation of membrane proteins (Carpenter & Cohen, 1979; Carpenter et al., 1979; Ushiro & Cohen, 1980), and changes in the cytoskeleton (Schlessinger & Geiger, 1981) and in cell morphology (Chinkers et al.,

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[1] Abbreviations: EGF, epidermal growth factor; R-EGF, EGF labeled with tetramethylrhodamine; EGF-RC, EGF receptor complex; FPR, fluorescent photobleach recovery; PBS, phosphate-buffered saline; WW591, 5-[4-[3-(γ-sodium sulfopropyl)-6,7-benzo-2(3*H*)-benzoxazolylidene]-2-butenylidene]-1,3-dibutyl-2-thiobarbituric acid; diI, 3,3′-dioctadecylindocarbocyanine iodide.

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1979). It also stimulates cell proliferation in vitro and in vivo (Carpenter & Cohen, 1979) by unknown mechanisms.

EGF binds to diffusely distributed (Schlessinger et al., 1978a; Haigler et al., 1978, 1979; Schlessinger, 1980) laterally mobile membrane receptors which rapidly form visible patches in a temperature-dependent process and which become endocytosed (Schlessinger et al., 1978b; Gorden et al., 1978) and degraded by lysosomal enzymes (Carpenter & Cohen, 1976).

McKanna et al. (1979) bound ferritin–EGF to human epidermoid carcinoma cells (A-431), a cell line which is unusually rich in EGF receptors, and showed by electron microscopy that at 4 °C EGF receptors are primarily diffusely distributed on the cell surface. When the cells were heated, microclusters containing 10 or more ferritin–EGF molecules are formed primarily over coated regions of the plasma membrane. These microclusters are subsequently endocytosed and concentrated into multivesicular bodies. Using an eosin derivative of EGF, Zidovetzki et al. (1981) measured the rotational relaxation of the EGF–(EGF receptor) complexes (EGF–RC) on viable A-431 cells using the method of time-resolved phosphorescent emission and anisotropy (Austin et al., 1979). At 4 °C the EGF-RC's were mobile with a rotational relaxation time of 10–50 μs which corresponds to the free rotation of single EGF-RC molecules in a fluid lipid matrix. The relaxation time increased progressively with temperature, and it was suggested that EGF-RC microclusters were being formed (Zidovetzki et al., 1981). Moreover, these microclusters maintained their dynamic independence even after they merged to form visible patches. It was argued previously that the formation of microclusters of EGF receptors is required for the induction of DNA synthesis and for the processing of the membrane-bound EGF receptors (Shechter et al., 1979; Schlessinger, 1980).

Patch formation and receptor-mediated endocytosis are temperature-dependent processes which are completely inhibited at 4 °C (Silverstein et al., 1977; Goldstein et al., 1979; Brown & Goldstein, 1979; Schlessinger, 1980; Haigler et al., 1979). If receptor-mediated endocytosis occurs at specific sites on the cell surface (i.e., coated regions), its inhibition at low temperatures could be due to the decreased lateral diffusion of the occupied EGF receptors. Similarly, if patch formation occurs on the cell surface, its inhibition at low temperatures could also be due to the decreased lateral diffusion of the EGF-RC. To test these possibilities, we have measured the lateral diffusion of rhodamine-labeled EGF bound to human epidermal carcinoma A-431 cells as a function of temperature. We will show that lateral diffusion is not rate limiting in either case. Furthermore, we will present more evidence that the patching of fluorescent EGF occurs inside the cell. Finally, we will discuss the lateral diffusion of the lipid-soluble probes and the EGF-RC as a function of temperature in terms of models for diffusion in eukaryotic membranes.

Materials and Methods

Cells. Human epidermoid carcinoma cells (A-431) were obtained from Dr. G. Todaro and were grown in 35-mm culture dishes in RMPI containing 10% horse serum, 5% fetal calf serum, and 100 units/mL penicillin–streptomycin under an atmosphere of 8% CO_2 and 92% air. For microscopic studies, the cells were grown on glass cover slips.

Reagents. EGF was purified from male mouse submaxillary glands according to the procedure of Savage & Cohen (1972). EGF was labeled with tetramethylrhodamine isothiocyanate (Shechter et al., 1978) or with iodine-125 (Hunter & Greenwood, 1962) according to published procedures. 3,3'-Dioctadecylindocarbocyanine iodide (diI) was the gift of Dr. A. Waggoner (Derzko & Jacobson, 1980) and 5-[4-[3-(γ-sodium sulfopropyl)-6,7-benzo-2(3*H*)-benzoxazolylidene]-2-butenylidene]-1,3-dibutyl-2-thiobarbituric acid (WW591) was the gift of Dr. A. Grinvald (Gupta et al., 1981).

EGF Binding. EGF or its derivatives were bound to the cells in either phosphate-buffered saline (PBS) containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS+) or a solution consisting of 4 parts of RPMI and 1 part of 50 mM Tris and 100 mM NaCl and 0.1% BSA adjusted to pH 7.4 (solution A). Cells were chilled on ice, washed twice with ice-cold PBS, incubated on ice with either R-EGF or [^{125}I]EGF (5 ng/mL) for 1 h in the appropriate buffer, and then washed 3 times with ice-cold PBS+.

Labeling of the Cells with Fluorescent Lipid Probes. WW591 dissolved in ethanol to an OD of 1.6 at 500 nm was dried under a stream of nitrogen. The WW591 was redissolved in 1 mL of PBS and vortexed. Cells were washed twice with PBS, and 0.95 mL of PBS was added either at room temperature or ice cold. Then 0.05 mL of the WW591 solution was added, and the cells were incubated for 15 min at room temperature or 45 min on ice. The cells were then washed twice with PBS at the appropriate temperature and used for microscopic studies.

We dissolved 0.3 mg of diI into 1 mL of ethanol and added 10 μL to washed cells immersed in 1 mL of PBS either on ice or at room temperature. After 10 min the cells were washed twice with PBS and used for microscopic studies.

Kinetics of the Internalization of [^{125}I]EGF. We bound [^{125}I]EGF to A-431 cells in solution A in the cold as described above. After washing the cells twice with ice-cold PBS+, we added 0.8 mL of solution A to the cells at a prescribed temperature and then immediately placed the dish into an incubator at that temperature. After incubating each dish for a given interval, we removed the solution A and added 0.2 mL of 50% trichloroacetic acid (Cl_3AcOH) to it, and to the cells we added 0.5 mL of an ice-cold solution of 200 mM acetic acid and 150 mM NaCl (solution B). After 6 min we removed solution B, rinsed the mixture with another 0.5 mL of ice-cold solution B, and pooled them. This treatment released the EGF bound to cell surface receptors (Haigler et al., 1980a; Yarden et al., 1981). We then dissolved the cells in 0.2 N NaOH. The radioactivity found here represented internalized but not yet released (due to complete degradation) EGF. The radioactivity in the Cl_3AcOH supernatant represented degraded EGF that had been internalized (Haigler et al., 1980; Yarden et al., 1981).

Fluorescent Photobleach Recovery and Image Intensification Microscopy. The lateral diffusion coefficient of the occupied EGF receptors was measured by the fluorescence photobleaching recovery (FPR) method (Axelrod et al., 1976; Schlessinger et al., 1976; Edidin et al., 1976; Jacobson et al., 1976). We have added to the FPR apparatus a silicon intensified target camera. Using this camera, we can localize even faintly staining fluorescent reagents on the cell. We also used this camera to align and focus the laser beam (Argon; 514 nm) on the cell surfaces. After the laser beam was focused, we applied a brief (10 ms) intense pulse which irreversibly bleached the fluorescence in a small region on the cell surface. The time course of recovery of fluorescence in the bleached region by fresh fluorophores from adjacent regions of the cell membrane was recorded. Diffusion coefficients (D; cm^2/s) were calculated from the FPR curves as previously described (Axelrod et al., 1976).

We regulated the specimen temperature during the experiment by using a Bailey Instruments SR-2 (Saddlebrook, NJ) microscope stage which exploits the Peltier effect for heating

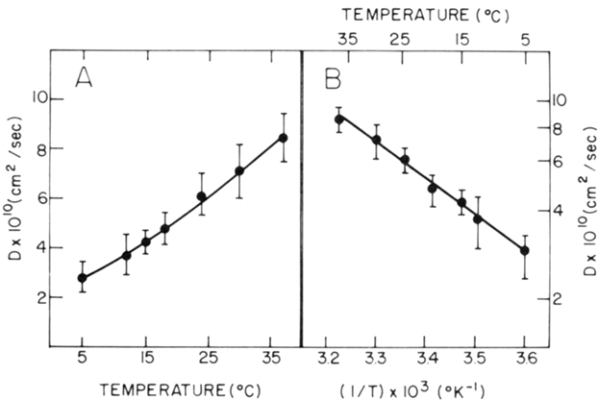

FIGURE 1: Lateral diffusion of R-EGF as a function of temperature. (A) A-431 cells grown on cover slips were labeled with 5 ng/mL R-EGF at 4 °C for 1 h, and the lateral diffusion coefficient, D, was measured at the appropriate temperatures by the fluorescent photobleach recovery technique. Eight to twelve measurements were taken at each temperatures, and the bars represent the standard error. (B) An Arrhenius plot of the data in (A). The apparent E_a is 6.1 kcal/mol.

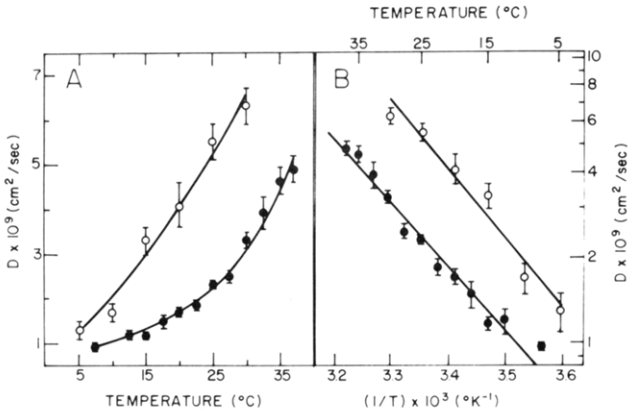

FIGURE 2: Lateral diffusion of the fluorescent lipid probes WW591 and diI. (A) A-431 cells grown on cover slips were labeled with WW591 (●) or diI (○) at room temperature or on ice, and their lateral diffusion coefficients were measured as a function of temperatures by the fluorescent photobleach recovery technique. An average of 20 measurements were made at each temperature, and the bars represent the standard error. (B) An Arrhenius plot of the data in (A). The apparent E_a is 10.3 kcal/mol for the WW591 and 11.2 kcal/mol for the diI.

and cooling. A microscope slide was preheated or precooled on the stage to the desired temperature and then a washed cover slip with the cells was mounted on the slide with 0.01 mL of washing buffer. Two minutes was allowed for equilibrium before any measurements were made. If we wished to investigate a different temperature with the same specimen, we changed the stage temperature and allowed 5 min for equilibrium. The specimen temperature was calibrated with an iron–constantan thermocouple sealed and sandwiched between a slide and a cover slip and mounted on the stage under experimental conditions.

Results

Thermal Dependence of the Diffusion of R-EGF and a Lipid Probe on A-431 Cells. The lateral diffusion coefficient of R-EGF bound to A-431 cells was measured by FPR techniques (see above) as a function of temperature (Figure 1A). All measurements were made before the onset of visible patches. The lateral diffusion coefficients increased gradually from 2.8×10^{-10} cm^2/s at 5 °C to 8.5×10^{-10} cm^2/s at 37 °C. The lateral diffusion coefficients of the lipid probes, WW591 and diI, were also measured by the FPR technique as a function of temperature (Figure 2A).

For WW591, D increased from 9.0×10^{-10} cm^2/s at 7.5 °C to 4.9×10^{-9} cm^2/s at 37 °C, while for diI it increased from 1.3×10^{-9} cm^2/s at 5 °C to 6.3×10^{-9} cm^2/s at 30 °C. No discontinuities were observed in any of the curves. Arrhenius plots of the data (Figures 1B and 2B) yielded apparent activation energies of 6.1 kcal/mol for the bound R-EGF, 10.3 kcal/mol for WW591, and 11.2 kcal/mol for diI.

No temperature-dependent trends were apparent in the mobile fractions for R-EGF bound to A-431 cells and for the lipid probes. The percent recoveries and standard deviations for R-EGF, WW591, and diI over the entire temperature range were 61 ± 18, 73 ± 13, and 84 ± 9. There were 35 measurements for R-EGF and diI and 80 for WW591. We emphasize that the samples were studied before the onset of visible patch formation (i.e., within 3 min for 37 °C, 7 min for 30 °C, and so on; see Figure 5) in order to minimize immobilization due to endocytosis and patching.

Cellular Location of Patches of R-EGF. A-431 cells were incubated for 15 min at 37 °C with 5 ng/mL R-EGF. Viewed by fluorescence microscopy, these cells appeared heavily patched and also showed a ring of diffuse membrane staining around their periphery (Figure 4C). We then chilled the cells

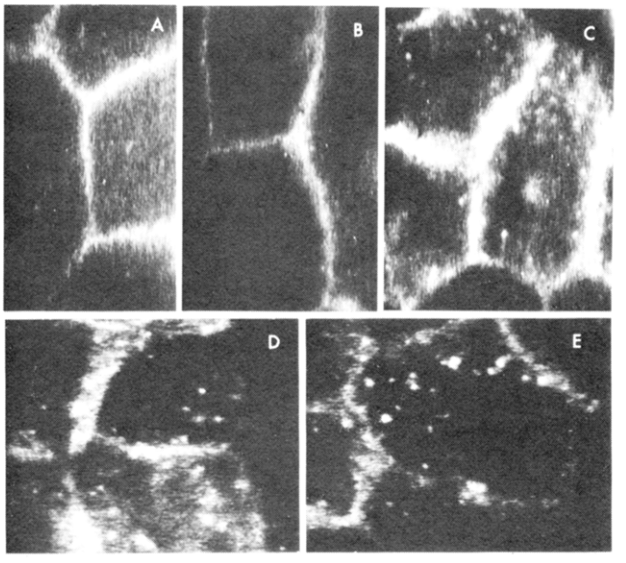

FIGURE 3: Formation of R-EGF patches. A-431 cells grown on cover slips were labeled for 1 h with 5 ng/mL R-EGF on ice. After being washed, the cells were placed on a slide preheated on the temperature-controlled microscope stage to 37 °C (time = 0), observed at 37 °C under an image-intensified fluorescent microscope, and photographed with a time-lapse video camera. (A) 2 min; (B) 4 min; (C) 5 min; (D) 6 min; (E) 10 min. Magnification: ×731.

and incubated them with acetic acid (see Materials and Methods), a treatment which releases more than 90% of the surface-bound [^{125}I]EGF but almost none of the internalized [^{125}I]EGF (Haigler et al., 1980; Yarden et al., 1981). Only patches remained (Figure 3D).

Thermal Dependence of Visible Patch Formation. We bound R-EGF (5 ng/mL) to A-431 cells at 4 °C for 1 h, washed the cells, and measured the time until patches began to form by recording their images at given temperatures with the image-intensified video fluorescent microscope. While the time for the onset of patch formation at 37 °C was 3 min, at 15 °C it was 75 min and at 4 °C it did not occur even after 6 h (Figure 4). The process was recorded by time-lapse video photography, and a sequence taken at 37 °C is shown in Figure 5. The data fit an Arrhenius plot of the reciprocal onset time with a correlation of −0.994; the apparent activation energy for patch formation was 26 kcal/mol.

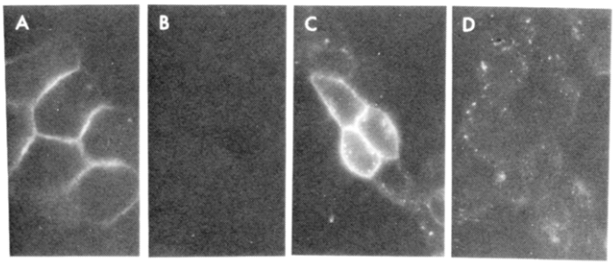

FIGURE 4: Location of R-EGF patches. A-431 cells were labeled with 5 ng/mL R-EGF for 1 h on ice (A and B) or for 15 min at 37 °C (C). After being washed with cold PBS+, the cells were treated for 6 min with an ice-cold solution containing 200 mM acetic acid and 150 mM NaCl in order to remove surface-bound R-EGF (B and D). The fluorescent preparations were observed with a Zeiss inverted microscope, IM-35, equipped with a filter set for the selective observation of rhodamine. Photographs were taken with Kodak Tri-X film. Magnification: ×329.4.

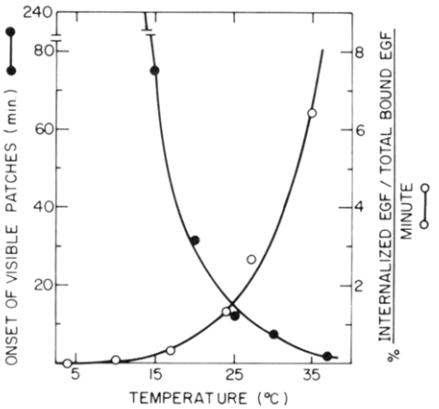

FIGURE 5: Kinetics of R-EGF patch formation and [^{125}I]EGF internalization. Patch formation: A-431 cells grown on cover slips were washed, incubated for 1 h at 4 °C with R-EGF (5 ng/mL), washed, mounted on a microscope slide which had been preequilibrated to the appropriate temperature on the temperature-controlled stage, and observed with an image-intensified fluorescent microscope; the time for the first appearance of the patches was recorded (●). Internalization: A-431 cells grown in 35-mm Petri dishes were washed and incubated for 1 h with [^{125}I]EGF (5 ng/mL) on ice, washed again, and then incubated at the indicated temperature. At regular intervals the medium from duplicate dishes was removed and treated with 12.5% Cl_3AcOH to separate degraded EGF from released intact EGF, and the cells were chilled and treated with acetic acid to remove surface-bound [^{125}I]EGF (see Materials and Methods). The cells were then dissolved in 0.2 N NaOH, and the internalized [^{125}I]EGF was determined. The initial rate of internalization was determined from plots of the ratio of the internalized plus degraded [^{125}I]EGF to the total bound [^{125}I]EGF vs. time, and it is plotted as a function of temperature (○).

Effect of Temperature on the Rate of [^{125}I]EGF Internalization. In a study similar to the onset of patching experiment, A-431 cells were labeled for 1 h with [^{125}I]EGF (5 ng/mL) at 4 °C, washed, and then incubated for varying periods at 0, 10, 17, 24, 27, 35, and 37 °C. The [^{125}I]EGF bound to the cell surface, internalized, degraded, and dissociated was measured for each point. The ratio of internalized (including degraded) EGF to total cell-associated EGF was determined for each temperature as a function of time. A plot of the initial velocity of internalization as a function of temperature is shown in Figure 5. The initial velocities fit an Arrhenius plot with a correlation coefficient of −0.992, and the apparent activation energy was 33.1 kcal/mol. This value is larger than those reported for the internalization of asialoorosomucoid by rat hepatocytes [15.6 kcal/mol (Weigel & Oka, 1981)] and α_2-macroglobulin by rabbit alveolar macrophages [19.5 kcal/mol (Kaplan & Nielsen, 1979)].

Discussion

Effect of the Value of D on Patching and Endocytosis. Our goal is to understand the processes which control hormone-induced receptor clustering. Therefore, we have characterized the dynamic properties of the EGF-RC that might affect the process of EGF-induced receptor clustering. Since the EGF-RC is internalized primarily via coated pits, if the migration of the EGF-RC to them is slowed at low temperatures, then this might inhibit patching and endocytosis. We chose to study A-431 cells because they are rich in EGF receptors which can be easily visualized by fluorescence microscopy after binding R-EGF. From the standpoint of lateral diffusion the A-431 cells behaved similar to normal cells. The diffusion coefficient of R-EGF bound to A-431 cells (4.7×10^{-10} cm^2/s at 20 °C) was similar to that observed in normal mouse 3T3 cells [3.4×10^{-10} cm^2/s at 22 °C (Schlessinger et al., 1978a)], and the fluorescent recovery of the R-EGF bound to A-431 cells (61%) was similar to that observed in 3T3 cells (50–85%) at room temperature (Schlessinger et al., 1978a). Also, the diffusion coefficient of dil in A-431 cells (5.5×10^{-9} cm^2/s at 25 °C) was comparable to its diffusion coefficient in myoblasts [8×10^{-9} cm^2/s at 25 °C (Schlessinger et al., 1977a)]. Since there is a 100-fold difference in the rate of internalization (and also of patching) between 10 and 37 °C (Figure 5) (they are completely inhibited at 4 °C), one might expect a large difference in the rate of diffusion. A large change in the diffusion coefficient often accompanies a phase transition (Wu et al., 1977; Vaz et al., 1981). No phase transition was observed (Figures 1 and 2) for either the EGF-RC or the lipid probes. The diffusion coefficient of the bound R-EGF increased gradually and only 3-fold from 5 to 37 °C (Figure 1).

Although the diffusion of proteins in biomembranes is often about 10 times slower than predicted (from the lipid viscosity) and also than observed in pure multibilayers (Cherry, 1979), it is still quite fast. To appreciate this, let us calculate the frequency of encounters between two EGF receptors. The area swept out by a receptor of diameter d after a displacement, s, over a time interval, t, is sd, and setting it equal to the reciprocal of the surface density, p, under the assumption that the receptors are evenly distributed on the cell surface, we have

$$1/p = sd \tag{1}$$

According to the classical theory of diffusion, the root mean squared displacement of lateral diffusion, s, equals $(4Dt)^{1/2}$ where D is the lateral diffusion coefficient. Substituting for s in eq 1 and solving for $1/t$, the encounter or collision frequency, we have

$$1/t = 4D(pd)^2 \tag{2}$$

Thus, the encounter frequency is linear with D. We have calculated encounter frequencies for EGF receptors with each other and with coated regions in an A-431 cell and in a human foreskin fibroblast or a 3T3 cell. The results are presented in Table I. The time required for all the receptors to encounter a coated region at 5 °C in an A-431 cell is 1.2 s and in fibroblasts, 8.8 s. The contour traveled by a diffusing molecule is substantially greater than its root mean square displacement. Therefore, our calculation is a lower bound to the collision frequency of the labeled receptors. While an encounter (collision) is not a reaction, it is clear that diffusion is not the rate-limiting step for patching and endocytosis at low temperatures.

On the basis of the measurements of the lateral and rotational (Zidovetzki et al., 1981) diffusion of EGF receptors, visualization by ferritin–EGF (Haigler et al., 1979), and the

Table I: Encounter Frequencies

cell line	temp (°C)	receptor–receptor encounters s^{-1} receptor^{-1}, $1/t$[a] (1/s)	receptor-coated region; time for all receptors to encounter a coated region, t/n[b] (s)
A-431[c]	5	0.51	1.2
A-431	37	1.6	0.4
fibroblast[d]	5	0.01	8.8
fibroblast	37	0.03	2.9

[a] t = encounter time (see the text); the number of receptor–receptor encounters per second per cell is given by $n/(2t)$ where n is the number of EGF receptors per cell. [b] To calculate the encounter frequency of the EGF receptors with the coated regions, we assumed that the receptors are fixed and evenly distributed (with surface density, p) on the cell surface and that one large, square coated region (whose dimensions are $d \times d$ and whose area is 2% of the cell's surface area) diffuses with the same D as a receptor. Then the encounter frequency per cell is given by $1/t = 4D[0.02(\text{cell area})]p^2$ and the time for all the EGF receptors to encounter a coated region is t/n where n is the number of EGF receptors per cell. [c] From the electron microscopic studies of Haigler et al. (1979), the surface area of an A-431 cell is 2800 μm^2. It expresses one million EGF receptors whose half-maximal diameter is 6 nm. Our measured diffusion coefficients were $D(5\ °C) = 2.8 \times 10^{-10}$ cm^2/s and $D(37\ °C) = 8.5 \times 10^{-10}$ cm^2/s. [d] Human foreskin or mouse 3T3 fibroblasts. Their surface area is 1000 μm^2 and they express 50 000 receptors. Their receptor size and mobility are assumed to be the same as in A-431 cells.

determination of the rate of internalization of radiolabeled EGF and the onset of visible patching of fluorescent EGF, it is possible to draw an integrated picture of the dynamic properties of EGF receptors. At 4 °C, EGF receptors translate and rotate rapidly in the plane of the membrane of A-431 cells. At higher temperatures EGF receptors form clusters composed of 10–50 molecules (within 1–2 min at 37 °C). The microclusters are rapidly internalized, some through coated pits (Gorden et al., 1978; Haigler et al., 1979), into endocytotic vesicles.

Since the internalization of radiolabeled EGF receptors precedes the formation of "visible patches", it is reasonable to assume that the bright patches observed in fluorescence microscopy were endocytotic vesicles formed by the coalescence of several vesicles, each containing microclusters of EGF receptor complexes.

Models for Lateral Diffusion in Membranes. While the lateral diffusion coefficient for the EGF-RC increased upon heating from 4 to 37 °C, the rotational correlation relaxation time decreased in the same range (Zidovetzki et al., 1981). According to the theory of Saffman & Delbruck (1975), the rotational diffusion coefficient is sensitive to the radius of the rotating species while the lateral diffusion coefficient is relatively insensitive to the diameter of the diffusion entity. Moreover, both the lateral diffusion and the rotational diffusion should increase with temperature as a function mainly of $T/\eta(T)$ where T is the absolute temperature and $\eta(T)$ is the temperature-dependent viscosity of the membrane. However, if microclusters are formed upon heating to 37 °C, the retardation contributed by the clustered receptors could outweigh the contribution due to increased kinetic energy and decreased membrane viscosity. Then the newly formed microclusters would rotate slower at 37 °C than individual receptor molecules at 4 °C (Zidovetzki et al., 1981). In contrast, since the lateral diffusion is relatively insensitive to size, both individual EGF receptors and microclusters would diffuse at comparable rates which increase with temperature. This is what was observed.

In pure phospholipid bilayers, the diffusion coefficient of either protein (Vaz et al., 1981) or lipid probe (Wu et al., 1977) changes abruptly by a 100-fold as a function of temperature in a gel-to-fluid phase transition. A 1:1 molar ratio of cholesterol to phospholipid abolishes the gel phase and, thus, the abrupt transition, and the lipid diffusion increases only 10-fold over the same range of temperatures (Wu et al., 1977). The observed gradual 3-fold increase for EGF-RC and the gradual 5-fold increase for the two lipid probes are consistent with the notion that both cholesterol and membrane protein abolish the gel-to-fluid phase transition in the plasma membrane of eukaryotic cells.

The diffusion of proteins in phospholipids is about 10 times faster than in biological membranes (Vaz et al., 1981). the retardation in the plasma membranes may be due to rapid binding and release of the EGF-RC with immobile structural elements in the plasma membrane or with cytoskeletal elements (Edelman, 1976; Schlessinger et al., 1977b; Geiger & Singer, 1979; Henis & Elson, 1981; Koppel et al., 1981). Furthermore, lateral phase separations and the partitioning of the receptor and the lipid probes into and out of gel-like domains may also cause the observed lateral diffusion to appear slower than it is (Shimshick & McConnell, 1973; Klausner & Wolf, 1980; Klausner et al., 1980; Smith et al., 1980; Hackenbrock, 1981).

One would predict from the theory of Saffman & Delbruck (1975) that D is proportional mainly to $T/\eta(T)$ (see above). However, in both artificial and biological membranes, ln D has been proportional to $1/T$. Thus, either the viscosity is a complicated function of temperature or other interactions contribute significantly to the observed lateral diffusion in membranes. It has been useful to calculate the apparent activation energy, E_a, from Arrhenius plots derived for the temperature dependence of lateral diffusion. The apparent E_a of the EGF-RC (6.1 kcal/mol) is similar to the apparent E_a calculated for the diffusion of proteins and lipids in artificial lipid bilayers. The apparent E_a for gramicidin S in EPC (egg lecithin) bilayers was 4.1 kcal/mol, and in bilayers containing a 0.5 molar ratio of cholesterol, it was 2.1 kcal/mol while with a 1:1 ratio it was 6.8 kcal/mol (Wu et al., 1978). The apparent E_a for diI in EPC bilayers was 3.6 kcal/mol (Derzko & Jacobson, 1980), while in A-431 cells E_a(diI) = 11.2 kcal/mol and E_a(WW591) = 10.3 kcal/mol. While it seems that other interactions beyond the viscosity of the lipid matrix control the diffusion of the EGF-RC, nevertheless, the apparent activation energy for diffusion is similar to the activation energy of proteins and lipids which diffuse in artificial bilayers.

This work, taken together with that of Zidovetzki et al. (1981), presents a comprehensive description of the dynamic behavior of the EGF-RC. Even though the EGF-RC diffuses rapidly (translate and rotate) at 4 °C on the membrane surface and encounters with coated regions and other receptors are frequent, only a few receptors have enough energy to form microclusters or bind to coated regions. Since energies of the EGF-RC's are distributed according to Maxwell's equation, at higher temperatures substantially more EGF-RC's have enough energy to form microclusters or bind to coated regions. Finally, it should be noted that while the internalization of coated vesicles is also a temperature-dependent process, there is no reason to believe that it is dependent only on the dynamic behavior of the EGF-RC's.

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