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Temperature-Dependent Lateral and Transverse Distribution of the Epidermal Growth Factor Receptor in A431 Plasma Membranes

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Summary. To elucidate further the structure and molecular dynamics of the epidermal growth factor receptor, temperature-dependent aggregation and extracellular protrusion of the epidermal growth factor receptor in isolated plasma membranes from A431 cells were examined by fluorescence energy-transfer techniques. Epidermal growth factor was labeled at the amino terminus with either fluorescein isothiocyanate or tetramethylrhodamine isothiocyanate. A radionuclide receptor displacement assay demonstrated the bioactivity of these derivatives. Aggregation of the epidermal growth factor receptor was measured by determining the increase in fluorescence energy transfer between receptor-bound fluorescein and tetramethylrhodamine-labeled epidermal growth factor. Energy transfer between receptor-bound fluorescent derivatives was reversibly greater at 37 than 4°C, indicating temperature-dependent aggregation of the receptor. The extracellular protrusion of the epidermal growth factor receptor was calculated from the magnitude of energy transfer between receptor-bound fluorescein labeled epidermal growth factor and 5-(N-dodecanoylamino)-eosin partitioned into the lipid membrane at 4 and 37°C. No significant change in the distance of closest approach between the N-terminus of epidermal growth factor and the plasma membrane was observed at 4°C (69 ± 2 Å) and 37°C (67 ± 2 Å). Thus, the extracellular protrusion of the occupied epidermal growth factor receptor did not change detectably upon receptor aggregation.

Key Words epidermal growth factor receptor · A431 cells · fluorescence energy transfer · receptor aggregation

Introduction

Epidermal growth factor (EGF)[1], a 53 amino acid polypeptide, is a potent mitogen for various epidermal and epithelial cells (Carpenter & Cohen, 1979). The effects of EGF are mediated through the EGF receptor, a M_r = 170,000 integral membrane glycoprotein found in the plasma membranes of most mammalian somatic cells (Carpenter & Zendegui, 1986). The cDNA nucleotide sequence encoding the human EGF receptor predicts a single chain 1186 amino acid polypeptide comprised of three domains: an extracellular domain, an intracellular domain, and a transmembrane domain (Ullrich et al., 1984). The 621 amino acid N-terminal extracellular domain can bind EGF with high affinity (Ullrich et al., 1984). The 541 amino acid intracellular domain possesses tyrosine kinase activity (Ullrich et al., 1984). This tyrosine kinase capacity is required for the EGF receptor to promote mitogenesis (Chen et al., 1987; Honegger et al., 1987; Moolenaar et al., 1988). The extracellular ligand binding domain is connected to the cytoplasmic tyrosine kinase domain by an approximately 23 amino acid transmembrane domain which traverses the plasma membrane once. A major focus of EGF receptor research is to determine how the mitogenic signal, initiated by EGF binding, is transduced across the membrane activating the cytoplasmic tyrosine kinase when only a minimal structure connects these two major domains of the receptor.

Both intramolecular (Weber, Bertics & Gill, 1984; Koland & Cerione, 1988); and intermolecular (Biswas et al., 1985; Basu et al., 1986; Schlessinger, 1986; Yarden & Schlessinger, 1987*a*) models for EGF-induced tyrosine kinase activation have been proposed. The intermolecular models are based upon conformational changes associated with the

[1] *Abbreviations:* EGF, epidermal growth factor; PBS, phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 7.9 mM Na_2HPO_4, (1.5 mM KH_2PO_4, 0.87 mM $CaCl_2$, 0.5 mM $MgCl_2$); C_{12}-eosin, 5-(N-dodecanoylamino)-eosin; C_{12}-fluorescein, 5-(N-dodecanoylamino)-fluorescein; BSA, bovine serum albumin; HEPES, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; FITC-EGF, N^{α}-fluorescein-5-thiocarbamoyl-asparagine-1-EGF; TRITC-EGF, N^{α}-tetramethylrhodaminethiocarbamoyl-asparagine-1-EGF; RP-HPLC, reverse-phase high-pressure liquid chromatography.

aggregation state of the EGF receptor. EGF-induced oligomerization has been demonstrated with various biochemical (Carpenter & Cohen, 1976; Fanger et al., 1986; Boni-Schnetzler & Pilch, 1987; Yarden & Schlessinger, 1987*a,b*; Cochet et al., 1988) and biophysical (Zidovetski et al., 1981, 1986; Hillman & Schlessinger, 1982; Carraway, Koland & Cerione; 1989) techniques. What conformational changes occur upon receptor aggregation and tyrosine-kinase activation or regulation are unclear.

In order to determine various structural and dynamic features of the EGF receptor, we examined the temperature-dependent aggregation and extracellular protrusion of the occupied EGF receptor in plasma membranes from A431 cells. The magnitude of fluorescence resonance energy transfer between receptor-bound fluorescein labeled EGF and 5-(N-dodecanoylamino)-eosin (C_{12}-eosin) partitioned into the lipid membrane was used to measured the distance from the extracellular protrusion of the EGF receptor to the plasma membrane. The observed increase in fluorescence energy transfer between receptor-bound fluorescein and tetramethylrhodamine-labeled epidermal growth factor assessed receptor aggregation. Although occupied EGF receptors reversibly move closer together at elevated temperatures, receptor aggregation did not cause the receptor in isolated plasma membranes to protrude either further in or out of the membrane. These results provide constraints for molecular models of the EGF receptor and its activation.

Materials and Methods

Materials

Murine EGF was purified (Savage & Cohen, 1972) from adult mouse submaxillary glands (Pelfreez), and radiolabeled with Na[^{125}I] (Amersham) to a specific activity of (1.0–1.3) $\times$ 10^{18} cpm/mol (Carpenter & Cohen, 1976). Fluorescein-5-isothiocyanate (Isomer I), 5-(N-dodecanoylamino)-eosin and 5-(N-dodecanoylamino)-fluorescein (C_{12}-fluorescein) were purchased from Molecular Probes (Eugene, OR). Tetramethylrhodamine isothiocyanate (Isomer R) was purchased from BBL (Cockeysville, MD). All cell culture reagents were obtained from Gibco (Grand Island, NY). All other chemicals were at least reagent grade.

Fluorescent Labeling of EGF

Fluorescent isothiocyanate labeling reagents (1.6×10^{-6} mole in dimethylformamide) were added to EGF (1.6×10^{-7} mole in 200 μl dimethylformamide) and pyridine (50 μl). After 4 hr of constant stirring at 22°C, the mixture was eluted through a P-2 (BioRad) column (0.9 $\times$ 90 cm) equilibrated with 50 mM Tris-HCl, pH 7.4, at 4°C. The excluded peak was chromatographed at room temperature by reverse-phase high performance liquid chromatography (RP-HPLC) by means of a Beckman Model 334 system. The sample was loaded in multiple 1.5 ml aliquots onto a Vydac C_4 (214TP54) column equilibrated with 50 mM acetic acid, pH 4.60. The polypeptides were separated with a 60-min linear gradient between 31 to 33% acetonitrile in 50 mM acetic acid, pH 4.60, eluted at 0.5 ml/min. The major fluorescent peak with EGF binding capacity was rechromatographed with the same gradient.

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis methods combined a discontinuous buffer system (Laemmeli, 1970) with the gel porosity formulations of Weber, Pringle and Osborn (1972). To the top of the 20% acrylamide separating gel, a 3.3% stacking gel was layered. All samples were reduced with 1% β-mercaptoethanol prior to electrophoresis. The electrode buffer was 25 mM Tris, 0.19 M glycine, pH 8.3, 0.1% sodium dodecyl sulfate.

Quantitation of Fluorescent EGF Derivatives

The protein concentration of the fluorescent EGF derivatives was determined (Lowry et al., 1951) with native EGF as the standard. The molar extinction coefficient of 1.87 $\times$ 10^4 (A_{280}) and relative molecular mass of 6045 were used to determine the native EGF concentration (Taylor, Mitchel & Cohen, 1972). Although fluorescein-5-isothiocyanate and tetramethylrhodamine isothiocyanate react with the colorimetric assay reagents, the color intensity produced is less than 5% of the intensity arising from equimolar concentrations of FITC-EGF or TRITC-EGF, respectively.

Cells and Cell Culture

A431-29i cells (Santon et al., 1986), generously provided by G.N.Gill, were grown in roller bottles with DME/F12 (1 : 1) containing 5% fetal bovine serum, $NaHCO_3$ (20 mM), HEPES (20 mM), penicillin G (10^5 unit/liter) and streptomycin sulfate (100 mg/liter).

Plasma Membrane Preparations

A431 plasma membrane-enriched vesicles were prepared by the scraping method (Carpenter, King & Cohen, 1979) except all buffers contained phenylmethylsulfonyl fluoride (1 mM), leupeptin (10 μM), aprotinin (0.05 TIU/ml) and EGTA (1 mM) (Boni-Schnetzler & Pilch, 1987). The harvesting buffer was calcium free and magnesium free. These A431 plasma membrane-enriched vesicles are hereafter referred to as A431 membranes. The A431 membranes were flash frozen in liquid nitrogen, and kept frozen at -70°C without thawing until used.

The binding assay described below was used to determine the concentration of EGF receptors in A431 membranes. [^{125}I]-iodo-EGF was diluted 50-fold with native EGF to allow estimation of the specific activity, and the concentration of the EGF was at least 70-fold greater than the concentration of EGF binding sites. A plot of specifically bound CPM as a function of the concentration of A431 membranes was prepared, and the concentration of EGF receptors was calculated from the slope of this plot and the specific activity of the chemically diluted [^{125}I]-iodo-EGF.

Filtration Assay for Binding of EGF to A431 Membranes

The binding reaction was performed in triplicate in a total volume of 500 μl containing A431 plasma membranes, [^{125}I]iodo-EGF, and native or fluorescently labeled EGF in 0.10% BSA in phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 7.9 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.87 mM $CaCl_2$, 0.5 mM $MgCl_2$). After incubation, the bound [^{125}I]iodo-EGF was determined by measuring the radioactivity remaining after the reaction mixture was filtered through Millipore filters (GVWP 025 00) and washed with 10 ml cold 0.10% BSA in PBS. Nonspecific binding was defined by the reaction mixture containing excess native EGF (10 μM).

Steady-State Spectroscopy

Steady-state fluorescence and UV/VIS absorption measurements were made with a Perkin-Elmer MPF-66 spectrofluorometer and a Perkin-Elmer Lambda 3B spectrophotometer, respectively. Steady-state anisotropy measurements were made with Melles Griot dichroic sheet polarizers placed in the path of the excitation and emission beams.

Energy-Transfer Parameters

The Forster critical distance, R_o, represents the distance at which transfer efficiency is 50% and is calculated from Eq. (1) (Berman, Yguerabide & Taylor, 1980)

$$R_o = 9.765 \times 10^3(\kappa^2 J Q n^{-4})^{1/6}. \quad (1)$$

The overlap integral, J, is normally calculated from the spectral overlap between the donor emission spectrum and the acceptor absorption spectrum and the relationship (Berman et al., 1980)

$$J = \Sigma I_D(\lambda)\varepsilon_A(\lambda)\lambda^4\Delta\lambda/\Sigma I_D(\lambda)\Delta\lambda \quad (2)$$

where $\varepsilon_A(\lambda)$ is the molar extinction of the energy acceptor and $I_D(\lambda)$ is the donor emission. Q denotes the donor quantum yield in the absence of acceptor and n represents the refractive index of the medium between donor and acceptor. For proteins, the refractive index is about 1.4 (Stryer, Thomas & Carlsen, 1982). κ^2, the orientation factor, accounts for the relative orientation of the donor emission and acceptor absorption transition dipoles. λ represents the wavelength in cm.

Quantum Yield Determination for FITC-EGF and 5-(N-dodecanoylamino)-Fluorescein

The quantum yields of FITC-EGF in PBS and C_{12}-fluorescein in ethanol were determined at 4 and 37°C by means of the ratio method (Chen, 1965) and sodium fluorescein in 0.10 N NaOH at 20°C (Q = 0.85) (Umberger & LaMer, 1945; Parker & Rees, 1960) and C_{12}-fluorescein in ethanol at 20°C (Q = 0.34) (Holowka & Baird, 1983*a*) as standards, respectively. The quantum yield of receptor-bound FITC-EGF was determined by observing the change in fluorescence as free FITC-EGF (10 nM) bound to A431 membranes (20 nM in EGF sites).

Temperature-Dependent EGF Receptor Aggregation in Plasma Membrane-Enriched Vesicles

The capacity of receptor-bound FITC-EGF to transfer energy to receptor-bound TRITC-EGF was used to assess changes in the aggregation of occupied EGF receptors. Where the acceptor is fluorescent, transfer efficiency can normally be estimated from measurements of either donor quenching or acceptor sensitization by exciting the donor and monitoring the emission at the donor or acceptor wavelengths, respectively. When the donor fluorescence-quenching was measured, the apparent efficiency of energy transfer, E_D, was calculated from Eq. (3) (Berman et al., 1980).

$$E_D = 1 - I_{DA}/I_D \quad (3)$$

where I_{DA} and I_D are the fluorescence intensities in the presence and absence of acceptor, respectively. When sensitized acceptor emission was measured, the apparent energy-transfer efficiency, E_A, was measured from Eq. (4) (Berman et al., 1980).

$$E_A = A_A/A_D(I_{AD}/I_A - 1) \quad (4)$$

where I_{AD} and I_A are the fluorescence intensities of the acceptor in the presence and absence of donor, respectively. A_A and A_D are the absorbances at the excitation wavelength of the donor and acceptor, respectively.

The magnitude of donor quenching and acceptor sensitization was determined from corrected emission spectra of the following samples bound to A431 membranes: (i) FITC-EGF (2 μM) and TRITC-EGF (4 μM), (ii) FITC-EGF (2 μM) and native EGF (4 μM), (iii) TRITC-EGF (4 μM) and native EGF (2 μM). All three samples were prepared by adding the ligands simultaneously to A431 membranes (21 nM in EGF sites) in PBS at 4°C. After incubation for 60 min, the free ligands were removed by centrifugation at 25,000 × g for 30 min at 4°C. Corrected emission spectra were measured of the pellets resuspended in PBS. One hour transpired between each change of temperature.

Distance of Closest Approach between the Membrane Surface and the N-Terminus of Receptor-Bound EGF

FITC-EGF (1.5 μM) was incubated with A431 membranes (10 nM in EGF sites) for 60 min at 4°C, followed by centrifugation at 25,000 × g for 30 min to remove the free ligand. The titration was performed in triplicate in 0.5 cm curvettes with C_{12}-eosin (590 μM in dimethylsulfoxide) added to the pellet resuspended in PBS. The total amount of dimethylsulfoxide added never exceeded 1.5%. Fluorescence from background and direct excitation of acceptor C_{12}-eosin were estimated from the titration of C_{12}-eosin into A431 plasma membranes in the absence of FITC-EGF. Energy transfer was measured as the extent of donor quenching after subtraction of background and correction for dilution and inner filter effects. Nonspecific energy transfer was defined as the energy transfer measured when FITC-EGF was prevented from specifically binding to the EGF receptor by excess native EGF (10 μM). Specific energy transfer was the difference between the total and nonspecific energy transfer.

Estimates of the relative distance of closest approach between the lipid membrane surface and the N-terminus of receptor-bound EGF were obtained from the slopes of plots of Q_D/Q_{DA}

versus σR_o^2. The slope $(\pi/2\,(R_o/L)^4)$ of these plots is related to the distance of closest approach by Eq. (5) (Shaklai, Yguerabide & Ranney, 1977; Holowka & Baird, 1983*b*)

$$Q_D/Q_{DA} = 1 + (\pi R_o^6/2)(1/L)^4\sigma \quad (5)$$

where Q_D is the donor fluorescence in the absence of the C_{12}-eosin and L is the distance of closest approach between donors and acceptors. σ represents the surface density (acceptors/unit area). Equation (5) assumes a model in which donors are randomly distributed in a plane at a fixed distance, L, away from a plane of acceptors at the lipid membrane surface. The model also assumes a low surface density of acceptor molecules, κ^2 equals 2/3, and $L > 1.68\ R_o$ (Shaklai et al., 1977).

Acceptor Probe Surface Density

The surface density of C_{12}-eosin in A431 plasma membranes was determined by the method of Holowka and Baird (1983*a*). Briefly, C_{12}-eosin was titrated into A431 plasma membranes (10 nM EGF sites) containing partitioned donor C_{12}-fluorescein (540 nM) while monitoring the donor fluorescence in the absence (Q_D) and presence (Q_{DA}) of C_{12}-eosin.The surface density of acceptor probes was calculated from Eqs. (6) and (7) (Wolber & Hudson, 1979) which assume distances of closest approach of 0.00 R_o and 0.25 R_o, respectively.

$$Q_{DA}/Q_D = 0.6463\exp(-4.7497\sigma R_o^2) + 0.3537\exp(-2.0618\sigma R_o^2) \quad (6)$$

$$Q_{DA}/Q_D = 0.6290\exp(-4.5752\sigma R_o^2) + 0.3710\exp(-1.9955\sigma R_o^2). \quad (7)$$

The surface density used was the mean of these two calculated values.

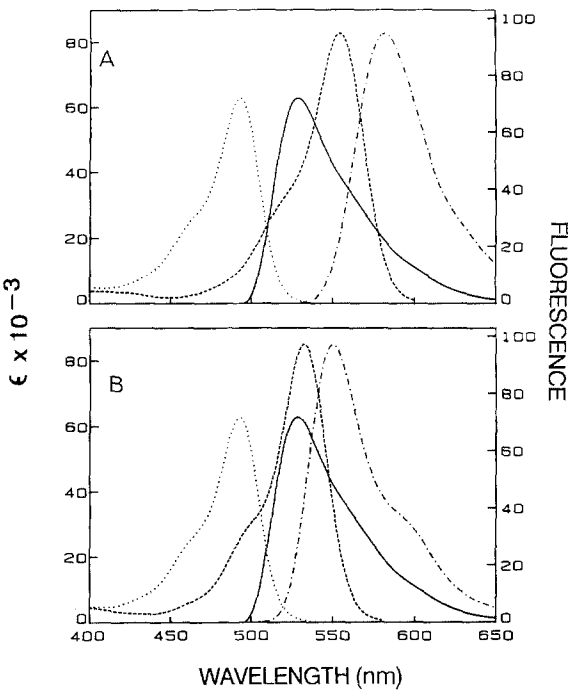

Fig. 1. Spectral overlap of fluorescent donor-acceptor pairs at 37°C. Shown are the absorption spectrum of free FITC-EGF (12 μM) (dotted) and corrected emission spectrum of receptor-bound FITC-EGF (16 nM) (solid) in *A* and *B*. *A* shows the corrected excitation spectrum (dashed) and emission spectrum (dot/dash) of receptor-bound TRITC-EGF (16 nM), and *B* shows the corrected excitation spectrum (dashed) and emission spectrum (dot/dash) of C_{12}-eosin (900 nM) partitioned into A431 membranes (9 nM in EGF receptors). All samples were suspended in PBS

Results

Purification and Biological Activity of Fluorescent EGF Derivatives

EGF could be site-specifically labeled at the N-terminal residue with isothiocyanate labels, because EGF is devoid of other primary amines such as lysine residues. To maximize the signal from the fluorescent EGF derivatives, the native EGF and excess free fluorophore were resolved by molecular exclusion chromatography followed by RP-HPLC. The RP-HPLC elution profiles of FITC-EGF and TRITC-EGF indicate only single peaks (*data not shown*). Native EGF could not be detected after the second RP-HPLC. Following sodium dodecyl sulfate electrophoresis of an aliquot of each of the two derivatives (~0.35 nmol), no nonconjugated TRITC or FITC could be detected (*data not shown*).

Binding was used to assay the biological activity of the fluorescent EGF derivatives. The capacity of FITC-EGF and TRITC-EGF to bind to EGF receptors in A431 membranes was measured with a competition assay. FITC-EGF and TRITC-EGF displayed approximately equivalent capacities to block [^{125}I]iodo-EGF from binding to A431 membranes. The IC_{50} values for FITC-EGF, TRITC-EGF, and native EGF were 29, 31, and 23 nM, respectively.

Spectral Characterization of EGF Derivatives

The spectral overlap of FITC-EGF/TRITC-EGF and FITC-EGF/C_{12}-eosin at 37°C are shown in Fig. 1. Corresponding spectra were also measured at 4°C (*data not shown*) and did not differ measurably from the spectra taken at 37°C. Because we could not directly measure the absorption spectrum of membrane-associated C_{12}-eosin, we measured the excitation spectrum of membrane-associated C_{12}-eosin and assumed the maximum extinction was equal to that for N-eosin-N′-phosphatidylethanolaminothiourea in phospholipid vesicles (85,000 $cm^{-1}M^{-1}$) (Fung & Stryer, 1978). The overlap integrals and

Table 1. Summary of energy-transfer parameters for receptor-bound donor FITC-EGF and acceptor TRITC-EGF

T (°C)	Q_D[a]	J[b] $\times 10^{13}$ (cm^{-6}/mol)	R_o[c] (Å)	E_D[d]	E_A[e]
4	0.39	3.8	53.1	0.00	0.06
37	0.34	3.8	51.9	0.06	0.21

[a] Quantum yield of receptor-bound donor FITC-EGF. [b] Overlap integral defined by Eq. (2). [c] Forster critical distance defined by Eq. (1) (assumptions: $\kappa^2 = 2/3$ and $n = 1.4$). [d] Efficiency of transfer from donor quenching measurements (Eq. (3)). [e] Efficiency of transfer by means of acceptor sensitization (Eq. (4)).

corresponding R_o values for the fluorophore pairs are listed in Table 1.

The quantum yields of receptor-bound FITC-EGF at 4 and 37°C were deduced from the measured quantum yields of free FITC-EGF at 4°C ($Q = 0.39$) and 37°C ($Q = 0.34$) and from the observation that the fluorescence of FITC-EGF did not change measurably upon binding (*data not shown*).

Temperature-Dependent EGF Receptor Aggregation in Plasma Membrane-Enriched Vesicles

To confirm that temperature-dependent aggregation occurs in isolated plasma membranes, receptor aggregation was assessed as a reduction of the distances between occupied receptors. Reduction of the distance between occupied EGF receptors was measured as an increase in fluorescence resonance energy transfer between receptor-bound FITC-EGF and TRITC-EGF.

The corrected emission spectrum depicting energy transfer from receptor-bound FITC-EGF to receptor-bound TRITC-EGF is shown in Fig. 2. For reference, a simulated emission spectrum of the sum of receptor-bound FITC-EGF and TRITC-EGF is also shown. The apparent efficiency of donor fluorescence-quenching increased from 0 to 6% after the cuvette was warmed from 4 to 37°C (Table 1). The apparent efficiency of energy transfer based on acceptor fluorescence sensitization also increased from 6 to 21% for the same temperature change. Receptor aggregation was reversible. Subsequent cooling of the cuvette from 37 to 4°C produced transfer efficiencies indistinguishable from those for the sample before warming to 37°C (*not shown*).

The apparent energy-transfer efficiency estimated as the extent of acceptor sensitization was greater than that estimated from the extent of donor quenching. This discrepancy might be due to the

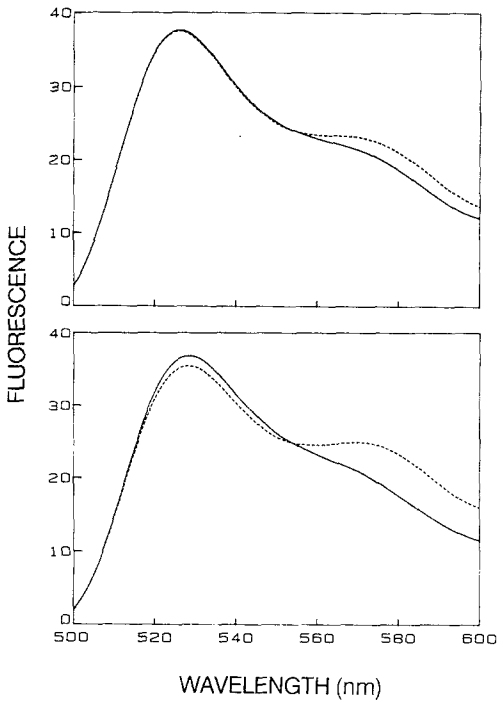

Fig. 2. Fluorescence resonance energy transfer between FITC-EGF and TRITC-EGF, bound to membrane associated EGF receptor. Shown are the corrected emission spectrum (EX: 450 nm) for a mixture (dashed) of receptor-bound FITC-EGF (7 nM) and receptor-bound TRITC-EGF (14 nM), and a simulated emission spectrum (solid) comprised of the sum of the separate spectra for receptor-bound FITC-EGF (7 nM) and receptor-bound TRITC-EGF (14 nM). Upper and lower panels show data for 4 and 37°C, respectively. For all spectra, a Corning 3-70 filter was placed in the path of the emission beam

absorbances of donor and acceptor at the excitation wavelength changing upon association with the receptor. Such a change would have distorted the calculated transfer efficiency (Eq. (4)). Due to the infeasibility of obtaining absorption spectra of turbid receptor-bound fluorescent ligands, the absorbances were estimated from excitation spectra of receptor-bound fluorescent ligands and the extinction coefficients for free ligands. Nevertheless, an increase in apparent efficiency of transfer from FITC-EGF to TRITC-EGF was observed at 37°C compared to 4°C regardless of the method of estimation.

While the efficiency of fluorescene energy transfer is commonly used to estimate intermolecular distances, the distance between the N-termini of adjacent receptor-bound EGF molecules was not determined. The calculation of intersite distances by means of dipolar fluorescence energy-transfer theory requires more information about the organization of EGF receptors in the plasma membrane than is currently available.

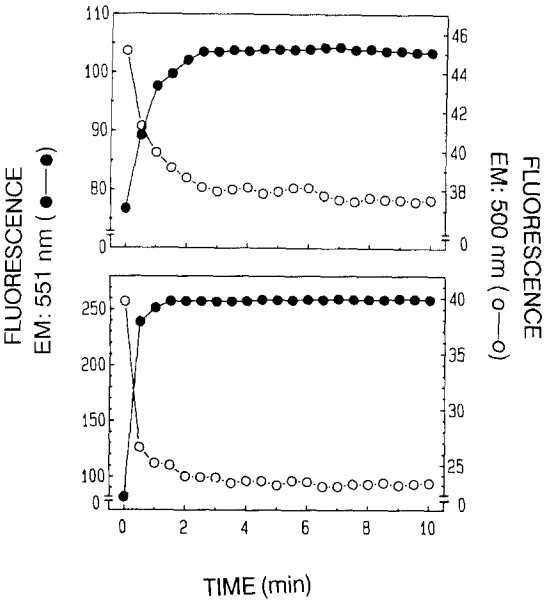

Fig. 3. Time-dependent partitioning of C_{12}-eosin into A431 membranes. The quenching of C_{12}-fluorescein fluorescence (EM: 500 nm) and concomitant sensitization of C_{12}-eosin fluorescence (EM: 551 nm) were monitored from corrected emission spectra (EX: 440 nm) taken at 30 sec intervals after C_{12}-eosin was added (to 950 nM) to A431 membranes (5 nM in EGF sites) containing partitioned C_{12}-fluorescein (200 nM). Upper and lower panels show data for 4 and 37°C, respectively. An Oriel 450 broad band filter was placed in the path of the excitation beam to reduce stray light effects

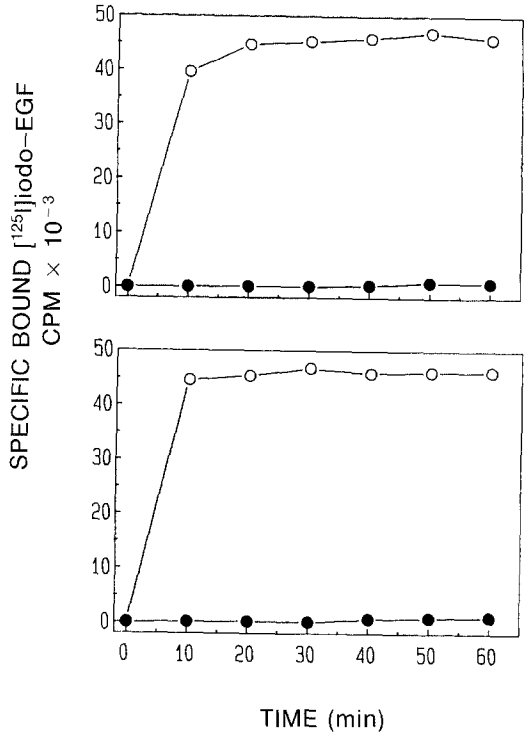

Fig. 4. Time-dependent displacement of receptor-bound FITC-EGF by [^{125}I]iodo-EGF. The specific binding of [^{125}I]iodo-EGF (11 nM) to A431 membranes (7 nM in EGF sites) was measured for receptor-bound FITC-EGF (7 nM) (filled circles) and buffer only (open circles). Upper and lower panels show data for 4 and 37°C, respectively

Distance of Closest Approach from the N-Terminus of Receptor-Bound EGF to the Membrane Surface

The distance of closest approach between the N-terminus of receptor-bound EGF and the lipid membrane surface was determined by means of another fluorescence resonance energy-transfer technique. The capacity of FITC-EGF to transfer energy to C_{12}-eosin partitioned into membranes was used to assess the relative distance of closest approach between receptor-bound FITC-EGF and the lipid membrane surface. This method involves titrating the fluorescent amphipathic probe C_{12}-eosin into A431 membranes. An initial study showed that the partition coefficient for this process is at least 0.95 at 22°C (*data not shown*). Furthermore, the time course of partitioning of C_{12}-eosin into A431 membranes was measured by monitoring both donor fluorescence-quenching and concomitant acceptor sensitization after C_{12}-eosin was added to A431 membranes containing partitioned C_{12}-fluorescein at 4 and 37°C. Figure 3 shows that after 5 min maximum partitioning of C_{12}-eosin occurred at both temperatures. Hence, all measurements in each step of the titration were taken 5 min after the titrant was added.

To ascertain whether the FITC-EGF was still bound to the EGF receptor after all steps of the titration, the time course of FITC-EGF displacement was measured. Figure 4 shows that after 60 min [^{125}I]iodo-EGF could bind to only 2.3 and 1.7% of the sites at 37 and 4°C, respectively. These results indicate that little FITC-EGF dissociates over the time course of the experimental measurements.

Because the determination of the surface density of acceptor molecules requires a knowledge of the Forster critical distance for the energy transfer from C_{12}-fluorescein to C_{12}-eosin, the temperature dependence of the Forster critical distance for this transfer was measured (*data not shown*). The relative quantum yield of C_{12}-fluorescein in ethanol and the overlap integral for spectral overlap between the fluorescence spectrum of membrane-partitioned C_{12}-fluorescein and the excitation spectrum of membrane-partitioned C_{12}-eosin were determined at 4° and 37°C. Because these parameters did not differ significantly, the published value of 50.1 Å (Holowka

Table 2. Summary of energy-transfer parameters for receptor-bound donor FITC-EGF and acceptor C_{12}-eosin

T (°C)	Q_D[a]	J[b] $\times 10^{13}$ (cm^{-6}/mol)	R_o[c] (Å)	Slope[d]	L[e] (Å)
4	0.39	3.6	52.6	0.546 ± 0.027	69 ± 2
37	0.34	3.6	51.4	0.560 ± 0.026	67 ± 2

[a] Quantum yield of receptor-bound donor FITC-EGF. [b] Overlap integral defined by Eq. (2). [c] Forster critical distance defined by Eq. (1) (assumptions: $\kappa^2 = 2/3$ and $n = 1.4$). [d] Linear regression slope of specific Q_D/Q_{DA} *versus* σR_o^2, ± standard error of regression slope. [e] Standard error based on 95% confidence interval ($t = 2.6$ for 5 degrees of freedom).

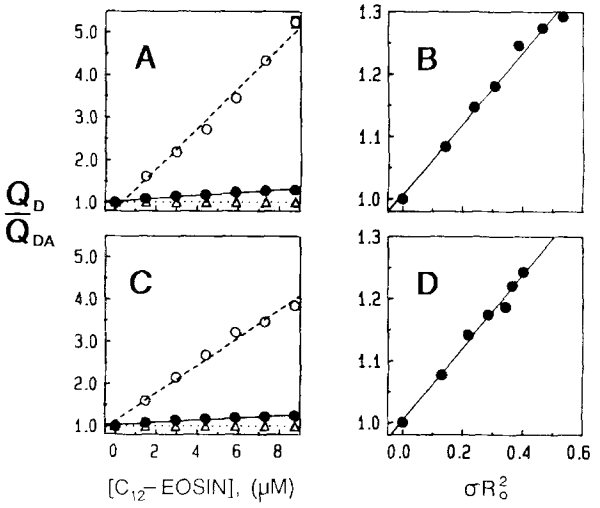

Fig. 5. Distance of closest approach between FITC-EGF and A431 membrane. *A* and *C* show the extent of donor fluorescence-quenching (EX: 450 nm with Oriel 450 broad band filter; EM: 510 nm with Corning 3-70 filter) from the titration of acceptor C_{12}-eosin into A431 membranes containing partitioned C_{12}-fluorescein (350 nM) (open circles), receptor-bound FITC-EGF (10 nM) (filled circles), and free FITC-EGF (10 nM) prevented from binding by prior incubation with excess native EGF (10 μM) (open triangles) at 4 and 37°C, respectively. *B* and *D* denote the ratio of donor quantum yield (specific fluorescence) in the absence (Q_D) and presence (Q_{DA}) of acceptor C_{12}-eosin as a function of acceptor density at 4 and 37°C, respectively

& Baird, 1983*a*) was used for estimating the acceptor C_{12}-eosin surface density at 4 and 37°C.

The distance of closest approach between the N-terminus of receptor-bound EGF and the lipid membrane surface was estimated from the C_{12}-eosin titration data (Fig. 5), the R_o values (Table 2), and Eq. (5). The slopes of the plots of specific Q_D/Q_{DA} as a function of σR_o^2 in Fig. 5*B* and *D* were determined by linear least-squares regression. The calculated distances of closest approach were 69 and 67 Å, for a nonaggregated and aggregated state of EGF receptors, respectively. Based on the standard error of the regression slope, an error range of ±2 Å was calculated with Student's t distribution with a confidence interval of 95% (Table 2). In a test for parallelism, slopes of normalized plots of Q_D/Q_{DA} *versus* σR_o^6 at 4 and 37°C (Figs. 5*B* and *D*) did not significantly differ ($t = 1.7$, $df = 10$, $P > 0.1$).

Discussion

With an in vitro system, two aspects of the molecular dynamics of the occupied EGF receptor were examined: temperature-dependent lateral and transverse movement. Occupied receptors were observed to move closer together as the temperature was raised from 4 to 37°C. This process was completely reversible, because subsequent lowering the temperature to 4°C restored the initial average interreceptor energy transfer. Also, no detectable aggregation-dependent change in the transverse distance between the receptor-bound EGF and the surface of the lipid membrane was observed.

Zidovetzki et al. (1986), measuring receptor rotational mobility, previously showed that the temperature-dependent aggregation of occupied EGF receptors was only partially reversible. We, on the other hand, observed complete reversibility of the temperature-induced aggregation. Why our results differ in this regard from Zidovetzki et al. (1986) is unclear. Two differences are worth noting. First, Zidovetzki et al. (1986) isolated plasma membranes from A431 cells in the presence of calcium and did not use protease inhibitors in the preparation of the A431 plasma membranes. Calcium-activated neutral proteases will covert the receptor to $M_r = 150{,}000$ polypeptide (Cassel & Glaser, 1982; Cohen et al., 1982), which may display different aggregation-dependent properties from the native $M_r = 170{,}000$ form. Second, additional temperature-dependent processes may occur which irreversibly affect the rotational mobility. The rotational diffusion measurements would detect the effect of these unknown processes, whereas fluorescence resonance energy-transfer measurements would not.

The process of receptor aggregation appears to be an intrinsic property of occupied EGF receptors. No exogenous sources of chemical energy were present in the plasma membrane samples to drive the aggregation reaction. Receptor aggregation and disaggregation occurred within 1 hr, the minimum time in which the temperature of the samples could be changed and spectroscopically measured. Be-

cause the aggregation reaction is more likely to occur at higher temperatures, the reaction involves positive enthalpy and requires positive entropy at 37°C. The molecular basis of this positive entropy is unclear. Elevation of temperature alone would normally increase the randomness of a system, decrease the dwell time of receptor-receptor complexes, and, consequently, reduce the association reaction. Lipid membrane phase changes would promote aggregation at the lower temperature as intrinsic membrane proteins might be excluded from ordered gel domains. The proteins excluded from ordered lipid domains might then aggregate. A likely explanation for temperature elevation to promote receptor aggregation would involve temperature-dependent conformational changes in the occupied EGF receptors. Although we cannot define these conformational changes, our results suggest that changes in the extracellular protrusion of the receptor probably do not occur upon aggregation.

Mindful of the precision of the transverse energy-transfer technique, discussed below, these results provide some constraints on the molecular models of the occupied EGF receptor. The distance between the N-terminus (Asn 1) of the receptor-bound EGF and the lipid membrane surface must be about 68 Å (Fig. 5 and Table 2). Although the orientation of EGF on the receptor is unknown, the N-terminus does not form part of the binding surface of EGF, because derivatization of the N-terminus of EGF with α-lactalbumin does not significantly affect binding to 3T3 murine fibroblast cells (Schecter et al., 1978).The large size of the α-lactalbumin (M_r = 14,500) relative to EGF (M_r = 6,045) suggests that the N-terminus of receptor-bound EGF is not close to the receptor.

Another feature of the EGF receptor that is suggested by our results is that, upon receptor aggregation, membrane lipids are not excluded from the aggregated receptors, because we observed no significant change in the energy transfer efficiency between the receptor-bound EGF and the C_{12}-eosin partitioned into the lipid membrane domain. If aggregation excluded lipids, there would have been a reduction in the FITC-EGF to C_{12}-eosin energy transfer. Consequently, domains of EGF receptor aggregates do not appear to exclude lipids.

Several considerations should be made in evaluating the above approach to assess transverse movement of the EGF receptor. These include (i) the precision of the approach, (ii) the resolving power of the approach to detect transverse movement, and (iii) effects of a possible reorientation of receptor-bound EGF.

The precision of the approach rests on several assumptions that require clarification. It should be noted that the usual uncertainty about the orientation factor, κ^2, is not a significant problem in these measurements, because the C_{12}-eosin has some rotational freedom. The rotational freedom of C_{12}-eosin is indicated, because the fluorescence anisotropy of C_{12}-eosin in the A431 membranes is significantly less (0.246 at both 4 and 37°C) than the anisotropy of eosin in a solution at infinite viscosity (0.340). Because of this rotational freedom, dynamic and probably some static averaging occur, and at the low transfer efficiencies, that we detected, the value of κ^2 is essential the same for dynamic and static averaging, $\kappa^2 = 2/3$ (Dale, Eisinger & Blumberg, 1979). Also, in calculating R_o, we utilized the excitation spectrum of membrane-associated C_{12}-eosin to estimate the profile of its absorption spectrum and assumed the maximum visible extinction equal to that for N-eosin-N′-phosphatidylethanolaminothiourea in phospholipids vesicles. Although the absorption spectrum of membrane-associated C_{12}-eosin could not be determined, large differences in the extinction of C_{12}-eosin would not affect the calculated distances. If, for example, the extinction of membrane-associated C_{12}-eosin was 50% of its value in ethanol, then the Forster critical distances would be about 46 Å, instead of about 52 Å, which would yield a 12% smaller value for the distance of closest approach.

We assumed that all occupied receptors aggregate. This may also be a reasonable assumption, because Zidovetzki et al. (1986) observed that the phosphorescence anisotropy decay curves at different temperatures of EGF-receptor complexes reasonably conformed to monoexponential behavior, which suggests that at each temperature most of receptors exist in the same state of aggregation. We assumed that the acceptor C_{12}-eosin randomly distributes on the surface of the membrane and the EGF receptor does not displace any of the membrane lipid. Although this assumption is strictly not correct, the amount of lipid displaced by the EGF receptor may be insignificant. The hydrophilicity of the extracellular and intracellular domains of the EGF receptor (Ullrich et al., 1984) suggest that only the transmembrane domain penetrates the lipid membrane, and, consequently, only a single α-helix with a cross-sectional area (<100 Å^2), equivalent to about one lipid molecule, would displace membrane lipids. Finally, the observed ratio of L/R_o was somewhat less (1.3) than the value (1.68) required by Eq. (5) to yield estimates with <5% error (Shaklai et al., 1977). The difference between the observed and the required L/R_o ratios is not large enough to invalidate the method, but does increase the uncertainty of the calculated distance of closest approach. Given the

above considerations a reasonable estimate of the uncertainty of the calculated distances of closest approach would be ±20%.

Separate from the precision of the transverse distance measurements is the issue of the resolving power of the transverse energy-transfer technique to detect relative transverse movement. Measurement of a change in the transverse distance involves fewer theoretical assumptions than measurement of the absolute transverse distance. Consequently, the major factor determining the uncertainty in the measurement of the changes in the transverse distance is experimental error. The experimental error in the present case gives ±2 Å resolution; consequently, we would detect transverse movements greater than 3 Å, if the distance of closest approach between FITC-EGF and the lipid membrane surface was about 68 Å.

Because of the random orientation of the donors and acceptors, shifts in the orientation of the fluorophores will have little effect on energy transfer; however, a transverse change in the orientation of the receptor-bound EGF molecular could occur independent of changes in the extracellular protrusion of the receptor. Such a movement could not be separated from transverse movements of the receptor.

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