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High affinity epidermal growth factor receptors on the surface of A431 cells have restricted lateral diffusion

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Rhodamine-labelled epidermal growth factor (Rh-EGF) was shown to bind to A431 cells grown at low density both to a small number of high affinity receptors (K_D = 2.8 x 10^{-10} M; fraction of total binding sites ~0.12) and also to a large number of low affinity receptors (K_D = 4 x 10^{-9} M; fraction of total binding sites ~0.88). Measurements of the lateral diffusion of EGF receptors on the cell surface were made using Rh-EGF and the technique of fluorescence photobleaching recovery. The high affinity receptors (labelled with 1.6 x 10^{-10} M Rh-EGF, 5% of EGF binding sites occupied) did not show lateral mobility over the temperature range 3° – 37°C. The low affinity receptors (labelled with 2.4 x 10^{-7} M Rh-EGF, 90% of EGF sites occupied) showed at least 75% fluorescence recovery after photobleaching, and lateral diffusion coefficients of ~2 x 10^{-10} cm²/s. These results show that the two populations of EGF receptors defined by binding studies differ in their freedom to diffuse laterally. The observation that the high affinity receptors are immobile indicates that lateral diffusion of receptors, at least over a distance of a few hundred nanometres or more, may not be required for the action of low concentrations of EGF.

Key words: epidermal growth factor/receptors/diffusion/cell surface

Introduction

Epidermal growth factor (EGF) binds to specific receptors in the plasma membranes of many epithelial and fibroblastic cell lines (for a survey, see Adamson and Rees, 1981) and in so doing initiates a cascade of intracellular reactions which ultimately results in stimulation of DNA synthesis and cell division. Immediately following the binding of EGF, clustering and internalisation of EGF-receptor complexes occur (Carpenter and Cohen, 1976; Schlessinger *et al.*, 1978a) accompanied by degradation of EGF (Carpenter and Cohen, 1976) and partial degradation of receptor (Das and Fox, 1978; Fox and Das, 1979). The precise roles of clustering, internalisation and degradation in EGF action remain uncertain for various reasons. It has been shown that, for promotion of target cells into S-phase, EGF must be present in the extracellular medium for a minimum of 6 – 8 h (Carpenter and Cohen, 1976; Rose *et al.*, 1975). The half-life of internalisation, however, is much shorter than this (30 – 60 min; Aharanov *et al.*, 1978; Krupp *et al.*, 1982) and the required presence of the growth factor during a period where the majority of receptors are absent from the cell surface (or in a state where they are unable to bind EGF) is therefore perplexing. Again, while experiments with CNBr-treated EGF and an anti-EGF receptor monoclonal antibody, 2G2IgM, have led to the hypothesis that clustering of receptors is an important prerequisite to biological action (see Schlessinger *et al.*, 1983), other results have demonstrated that clustering *per se* does not necessarily lead to the generation of a mitogenic signal (Stoscheck and Carpenter, 1983; Gregoriou and Rees, 1984).

EGF receptors occur in both high and low affinity states in many types of cell (MCF-7, a human breast cancer line, Osborne *et al.*, 1982; RAT-1, a rat fibroblast line, Magun *et al.*, 1980; HeLa, an epithelial line, Shoyab *et al.*, 1979; mink lung cells, Todaro *et al.*, 1976; C3H/2K, a mouse kidney line, Saito *et al.*, 1982; human skin fibroblasts, Krupp *et al.*, 1982; A431, a human epidermoid carcinoma line, Krupp *et al.*, 1982; Gregoriou and Rees, 1983; Kawamoto *et al.*, 1983). The maximum stimulation of DNA synthesis in responsive cells frequently occurs at EGF concentrations where most of the high affinity but very few of the low affinity sites are occupied. It may therefore be important to make the distinction between these sites when considering the relationship of receptor clustering and internalisation to the mitogenic action of the growth factor. One approach to resolving the question of clustering is to measure the rate of receptor movement in the plasma membrane under various conditions. This has been studied by Schlessinger and others in both mouse 3T3 fibroblasts (Schlessinger *et al.*, 1978b) and human A431 cells (Zidovetzki *et al.*, 1981; Hillman and Schlessinger, 1982), who observed that EGF-receptor complexes move laterally as a single diffusing species with D_L values in the range 2.8 x 10^{-10} cm²/s at 5°C to 8.5 x 10^{-10} cm²/s at 37°C. These experiments were performed with EGF concentrations of 10 nM on fibroblasts (Schlessinger *et al.*, 1978b) and 1 nM on confluent A431 cells (Hillman and Schlessinger, 1982). We have carried out similar experiments with the difference that sparse cultures of A431 cells were used so that lateral diffusion of EGF receptors on isolated single cells could be measured. Rhodamine-labelled EGF (Rh-EGF) was used both at a very low concentration (1.6 x 10^{-10} M) where mainly high affinity sites would be occupied and where stimulation of A431 cell growth has previously been observed (Kawamoto *et al.*, 1983) and at a relatively high concentration (2.4 x 10^{-7} M) where binding to low affinity sites would dominate the signal. The results of these experiments suggest that high affinity sites behave as a distinct class whose lateral mobility is considerably less than that of the major population of low affinity sites. Possible implications of this distinction will be considered in the Discussion.

Results

Purity of Rh-EGF

Rh-EGF, prepared as described in Materials and methods, was characterised by SDS-PAGE. The fraction from DEAE ion-exchange chromatography corresponding to Rh-EGF, as

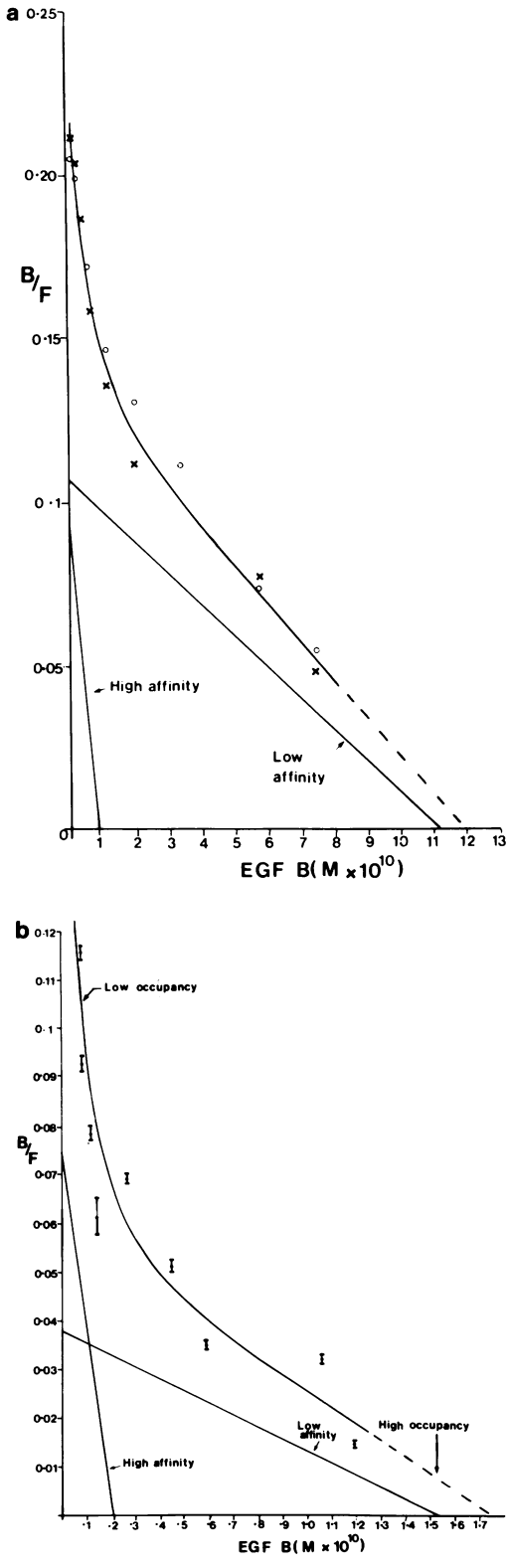

Fig. 1. Binding of EGF and Rh-EGF to A431 cells. (**a**) A Scatchard curve of EGF binding to semiconfluent A431 cells in which the receptor binding activities of native EGF (○) and Rh-EGF (x) are compared. The binding was carried out at 22°C and the curve is resolved into high and low affinity components. (**b**) A Scatchard curve of EGF binding to A431 cells at low density in which [^{125}I]EGF at 8 x 10^{-11} M is displaced with increasing concentrations of Rh-EGF. The binding was carried out at 22°C and is resolved into high and low affinity components. The points corresponding to the 'low occupancy' and 'high occupancy' fluorescence photobleaching experiments are indicated by vertical arrows. Error bars represent the range of values in a typical duplicate experiment.

described by Haigler *et al.* (1978), was run on a 20% gel and stained with Coomassie Blue. A parallel unstained sample was visualised by u.v. illumination and the only fluorescent band was directly superimposable on the Coomassie stained EGF which ran (typically) as a broad, diffuse band. No free rhodamine was detected.

Binding of Rh-EGF to A431 cells

To investigate the affinity of Rh-EGF, equilibrium binding assays were carried out on semiconfluent A431 monolayers (2.26 x 10^5 cells/cm^2) in 24-well multiwell plates. [^{125}I]EGF was bound to cells in the presence of varying amounts of unlabelled native EGF or Rh-EGF. The data, which were analysed by the Scatchard method (Figure 1a), shows that Rh-EGF binds to A431 receptors in an identical manner to native EGF. The curve obtained resolves into two components from which K_D values of 9.17 x 10^{-10} M (high affinity) and 1.04 x 10^{-8} M (low affinity) are obtained with an R_T (total receptor number) value of 1.9 x 10^6 sites per cell.

A separate series of assays was carried out to determine the values of these equilibrium binding parameters when A431 cells are at low cell density. This was necessary since the photobleaching experiment was carried out under these conditions. A Scatchard curve for low density cells is shown in Figure 1b and yields K_D values of 2.8 x 10^{-10} M (high affinity) and 4 x 10^{-9} M (low affinity) and an R_T of 1.84 x 10^6 sites per cell. Thus, although there is significant change in K_D of both high and low affinity sites with cell density, as shown by Gill and Lazar (1981) but assuming only a single class of sites, the total receptor number remains constant. From these data it is possible to estimate that, during the photobleaching experiments, when Rh-EGF was at a concentration of 1.6 x 10^{-10} M (hereafter referred to as 'low occupancy' labelling) the average percentage occupancy was 5.2%. Since ~7–12% of the total sites are high affinity at this cell density, low occupancy labelling would have been confined almost exclusively to high affinity sites. In contrast, when Rh-EGF was bound at 2.4 x 10^{-7} M, hereafter referred to as 'high occupancy' labelling, >90% of the total receptor population would be labelled.

Fluorescent labelling

It was important to demonstrate that cells labelled with Rh-EGF at a low occupancy of EGF binding sites (Rh-EGF at 1.6 x 10^{-10} M) had a fluorescence signal arising from the label that was well above that due to background or cell autofluorescence. This point is examined in Table I, where we list the fluorescence signals, measured with the laser-microscope in the photon counting mode, for cells exposed to differing concentrations of Rh-EGF. The main conclusions to be drawn from Table I are that: (i) cells labelled at low occupancy gave fluorescence signals 7-fold greater than that from the surrounding medium or from unlabelled cells; (ii) cells labelled with a mixture of 1.6 x 10^{-10} M Rh-EGF and 10^{-6} M unlabelled EGF did not fluoresce more than did unlabelled cells; and (iii) cells labelled at high occupancy gave a fluorescence signal 7- to 8-fold greater than that from cells labelled at low occupancy. We can conclude from these results that the fluorescence arising from labelling of high affinity receptors is clearly distinguished from cell autofluorescence. Furthermore, because the fluorescence signal arising from low occupancy labelling is abolished by excess unlabelled EGF, then the low occupancy Rh-EGF signal is not due to non-specific binding.

Table I. Fluorescence of A431 cells labelled with differing concentrations of Rh-EGF

Labelling conditions	Position of laser spot	Photon count rate (Hz) (mean, standard error of mean, number of cells observed)
No Rh-EGF	Cell	335 ± 39 (6)
	Background	190 ± 20 (6)
1.6×10^{-10} M Rh-EGF	Cell	2100 ± 275 (7)
	Background	300 ± 19 (6)
1.6×10^{-10} M Rh-EGF with 10^{-6} M EGF	Cell	400 ± 80 (6)
	Background	280 ± 21 (6)
2.4×10^{-7} M Rh-EGF	Cell	15 600 ± 2100 (6)
	Background	2000 ± 50 (6)

The count rates have been corrected for dark current which, because of instrumental design (Garland, 1981) does not contribute in the analogue measurements used for fluorescence photobleaching recovery. Photon measurements were made with the laser beam focussed to a spot of 10 μm diameter, sufficient to excite fluorescence from most of a whole cell. The photon count rate was independent of cell temperature.

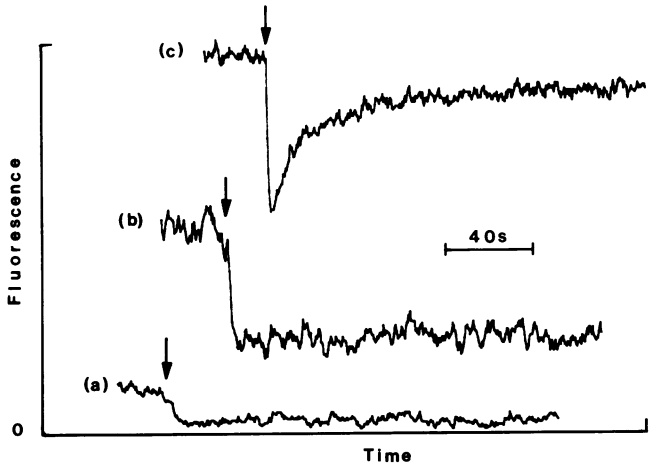

Fig. 2. Fluorescence recovery after photobleaching of Rh-EGF labelled A431 cells. The figure shows recorder tracings for three separate experiments. In trace **(a)**, which is a control for autofluorescence, labeling was with 1.6×10^{-10} M Rh-EGF plus 10^{-6} M EGF. In trace **(b)**, which is for high affinity sites, labelling was with 1.6×10^{-10} M Rh-EGF. In trace **(c)** labelling was with 2.4×10^{-7} M Rh-EGF and the fluorescence signal is predominantly from Rh-EGF bound to low affinity sites. The horizontal bar indicates the time scale. Fluorescence is in arbitrary units on a linear scale, the gain for traces **(a)** and **(b)** being five times greater than for trace **(c)**. Temperatures for these experiments were **(a)** 16°C, **(b)** 17°C, **(c)** 7°C. Photobleaching occurred at the points marked with vertical arrows.

Fluorescence photobleaching recovery

Figure 2 shows typical fluorescence photobleaching recovery curves for cells labelled at either low (1.6×10^{-10} M) or high (2.4×10^{-7} M) concentrations of Rh-EGF. Binding of Rh-EGF to the cell surface at low concentration of Rh-EGF was to high affinity receptors, as can be seen from the Scatchard plots of Figure 1. Significant (>10%) recovery of fluorescence after photobleaching for cells labelled at low occupancy of EGF receptors did not occur in the temperature range studied, from 3°C to 37°C, even if the measurements were continued for up to 20 min after photobleaching. Binding of Rh-EGF at the higher concentration was to both high and low affinity receptors, but the resulting fluorescence signal arose mainly from Rh-EGF bound to low affinity receptors because they are present in an ~8-fold excess over high affinity receptors (see Figure 1b). Fluorescence recovery in cells labelled at high occupancy of receptors by Rh-EGF was extensive (70–80% complete) and relatively fast ($t_{1/2}$ ~30 s) in the temperature range 3°–25°C. If such cells were kept at higher temperatures, 25–37°C, for several minutes, then the extent of fluorescence recovery fell, presumably because aggregation and internalization of receptors was occurring (Schlessinger *et al.*, 1978a,1978b; Zidovetski *et al.*, 1981).

If cell labelling was performed at a low concentration of Rh-EGF (1.6×10^{-10} M) mixed with an excess of unlabelled EGF (10^{-6} M), then the fluorescence signals were reduced several-fold compared with labelling with 1.6×10^{-10} M Rh-EGF alone (Figure 2, Table I). Although this autofluorescence signal could be irreversibly photobleached, its magnitude was too low to account for the irreversible bleaching seen with 1.6×10^{-10} M Rh-EGF alone and attributed to Rh-EGF bound to high affinity receptors.

Overall, the mean values (with standard error of the mean and number of individual cells observed in parentheses) for the fluorescence photobleaching recovery experiments were as follows. For high occupancy labelling the percentage recovery of fluorescence was 78% (±7, 20 cells) and D_L was 2.6×10^{-10} cm²/s ($\pm 0.45 \times 10^{-10}$, 20 cells). These mean values included measurements in the temperature range 3°–37°C, but excluded cells that had been held at the higher temperatures (>25°C) for >5 min. No attempt was made to determine a response of D_L to temperature, although a small effect has been reported (Zidovetski *et al.*, 1981; Hillman and Schlessinger, 1982). For low occupancy labelling the percentage recovery of fluorescence was zero, although signal-to-noise considerations indicated that small recoveries (>5%) would be difficult to detect. Physical stability of the position of the cell under observation is not maintained for more than 20 min or so. We cannot exclude diffusion with D_L values less than $\sim 10^{-13}$ cm²/s if it were assumed that complete recovery of fluorescence after photobleaching would have been observed for the low occupancy cells if only the measurement could have continued for long enough.

Discussion

The existence of a heterogeneous population of EGF receptors (high and low affinity sites) on A431 cells has been demonstrated by a number of laboratories (Kawamoto *et al.*, 1983; Krupp *et al.*, 1982; Gregoriou and Rees, 1983). While at high EGF concentrations (0.2–2 nM) A431 cell proliferation is inhibited due to a toxic effect (Gill and Lazar, 1981; Barnes, 1982; Gregoriou and Rees, 1984), when the concentration is reduced to 3–100 pM levels a growth stimulatory effect can be demonstrated (Kawamoto *et al.*, 1983). Many other types of cell exhibit affinity heterogeneity (see Introduction for a few examples) and for both human skin fibroblasts and mouse 3T3 fibroblasts, which respond normally to EGF when present in the range 1–2 nM, a particular role for the high affinity sites has been ascribed (Shechter *et al.*, 1978; and Collins *et al.*, 1983, respectively). The origin of the 'high affinity' argument lies partly in the fact that these sites represent only between 1 and 10% of the total receptor population,

depending on the cell type, and that the maximum mitogenic effect of many different growth factors is realised when a similar proportion of receptors is occupied. Thus, it is possible to assert that the low affinity population do not participate directly in eliciting the mitogenic response.

The inhibitory effect of EGF on A431 cells at concentrations where other cells respond maximally has, however, led to the conclusion that A431 cells are incapable of undergoing EGF-induced stimulation (Gill and Lazar, 1981; Willingham *et al.*, 1983; Lifshitz *et al.*, 1983). Since, at EGF concentrations in the range 0.8–1.5 nM, sparse A431 monolayers undergo down regulation with the loss of something like one million receptors per cell accompanied by severe membrane perturbation and vacuolisation, it may not be surprising that a toxic effect is observed. If, however, the EGF concentration required to give an equivalent level of receptor loss in A431 cells to that given by 1.5 nM EGF in normal cells (e.g., 3T3 cells, Aharonov *et al.*, 1978) is calculated, a figure somewhat less than 100 pM is obtained (from the data of Krupp *et al.*, 1982). Thus, the conditions for establishing a stimulatory rather than a toxic effect of EGF (or other growth factors) may derive from the need to occupy a large proportion of the high affinity sites while at the same time avoiding internalisation of a significant fraction of the plasma membrane protein compartment. Evidence for this type of interdependence can be found in the studies of Lifshitz *et al.* (1983) who demonstrated that, in cell clones derived from A431 but exhibiting differing levels of EGF receptors, inhibition was relaxed in those clones where the receptor number was low and was often accompanied by the ability to respond to EGF.

We have demonstrated that high affinity sites in A431 cells, which have previously been shown to mediate growth effects (Kawamoto *et al.*, 1983), can be distinguished from the major population of lower affinity sites by their considerably reduced rate of lateral diffusion. The difference between previous experiments (Hillman and Schlessinger, 1982) and those reported here is that, in the present study, measurements were carried out on sparse cultures of A431 cells and both very low (1.6×10^{-10} M) and very high (2.4×10^{-7} M) concentrations of Rh-EGF were used to study separately the high and low affinity receptors. The distinction between specific and non-specific binding of Rh-EGF was made possible by control incubations in which unlabelled EGF was also present. In addition, measurements at lower temperatures were made only when equilibrium binding had been attained (>5 h at 4°C). To demonstrate that the low D_L value (10^{-13} cm^2/s) obtained at low occupancy was not due to transfer of Rh-EGF-receptor complexes to an intracellular compartment, dissociation experiments were carried out in which [^{125}I]EGF at 1.6×10^{-10} M was bound to A431 cells at 4°C for 5 h at which time unlabelled EGF (5×10^{-8} M) was added and the cell-associated [^{125}I]EGF determined as a function of time. Full dissociation (>90%) occurred with a $t_{1/2}$ of 35 min. Thus, high affinity receptors on A431 cells are freely accessible to the extracellular medium, at least to a molecule the size of EGF. Contrasting mobility behaviour was shown by the low affinity receptors which diffused with a D_L value similar to that reported by others (Hillman and Schlessinger, 1982), that is, in the range $2-3 \times 10^{-10}$ cm^2/s.

The underlying molecular mechanism which might account for this biphasic diffusion behaviour is not known. Shechter *et al.* (1978) have reported that a class of EGF receptors in human fibroblasts experience persistent occupation even under dissociating conditions and have suggested as a result that high affinity sites can be distinguished from low affinity sites. More recent studies by King and Cuatrecasas (1982) on human KB cells have demonstrated similar behaviour of high affinity sites with the addition that they appear to be less protease-sensitive than low affinity sites. A large number of cases have now been reported in which factors unrelated to EGF are capable of removing the high affinity population of receptors in a non-competitive manner (e.g., PDGF, Collins *et al.*, 1983; FDGF, Rozengurt *et al.*, 1982; saccharin and cyclamate, Lee, 1981; phorbol esters, Shoyab *et al.*, 1979) possibly as a result of competition between receptors of different factors for a common effector molecule. Thus, slow lateral diffusion may be a consequence of association of some EGF receptors with immobile or slowly diffusing effectors. If this hypothesis is correct then the lateral mobility of high affinity receptors should increase in the presence of the type of non-competitive inhibitors described above. The possibility exists that the high affinity sites are those receptors already immobilised in coated pits although the recent results of Gregoriou and Rees (1984), who show that an antibody which promotes receptor clustering and internalisation actually reduces the affinity of the low affinity receptor population without affecting the high affinity population, militates against this mechanism.

In conclusion, we have demonstrated for the first time a physical distinction between high and low affinity receptors although we are not able to distinguish between models where either a small proportion of a homogeneous population associate with an effector molecule to give multiple classes in equilibrium or a heterogeneous receptor population pre-exists but responds independently and in different ways to EGF.

Materials and methods

Cell culture

Human epidermoid carcinoma A431 cells (Fabricant *et al.*, 1977) were grown to confluence in 25 cm^2 NUNC tissue culture flasks (Gibco, UK) in DMEM supplemented with 5% foetal-calf serum. For fluorescence measurements cultures were trypsinised and replated, in the above medium, into 90 mm Petri dishes (Sterilin, UK) containing sterile glass cover slips at a final cell density of 10^3 cells/cm^2. The dishes were left for 24 h at 37°C in an incubator (+5% CO_2) and cover slips were then taken for fluorescence measurements as described below.

Epidermal growth factor

Mouse EGF was a gift from Dr. H. Gregory (ICI Pharmaceuticals, UK). [^{125}I]EGF was prepared as described in Rees *et al.* (1979).

Rh-EGF was prepared as follows: EGF (1 mg, 0.17 μmol) was dissolved in 250 μl of redistilled dimethyl sulphoxide (DMSO) and 25 μl of an 18.2 mg/ml solution of rhodamine isothiocyanate (BDH Chemicals, UK) was added followed by a small volume (~1 μl) of N-ethyl morpholine to give an apparent pH of 8–9 (see Rees and Offord, 1976). After 4 h at room temperature, a further 25 μl were added and the reaction left for 12 h after which the solution was freeze-dried. The conjugated EGF was purified as described by Haigler *et al.* (1978) with the modification that the final ion-exchange step was followed by exhaustive dialysis against distilled water in Spectrapore 2000 (Pierce Chemical Co., USA). The EGF concentration was determined both by a radioligand binding assay (see Results) and by spectrophotometry using an E_{560} (mM) of 80 for rhodamine and an E_{280} (1 mg/ml) of 3.0 for EGF. Using these values the stock concentration of Rh-EGF was 19 μg/ml and the rhodamine to EGF ratio was 0.87 to 1.

Binding of [^{125}I]EGF to A431 cells

[^{125}I]EGF at 8×10^{-11} M and increasing concentrations of unlabelled native EGF or Rh-EGF over the range 1.5×10^{-10} M to 10^{-6} M (11-point assay) were added to A431 cells in 24-multiwell plates and allowed to come to equilibrium at either room temperature (2 h) or at 4°C (>6 h). Non-specific binding was taken as that [^{125}I]EGF bound to cells in the presence of 10^{-6} M unlabelled EGF and binding curves are shown after subtraction of this non-

specific component from each data point. Data were analysed by the method of Scatchard (1949).

Binding of Rh-EGF to A431 cells for fluorescence measurements

Rh-EGF was dissolved in Earles balanced salt solution (EBSS, without the addition of phenol but containing bovine serum albumin (BSA) at 5 mg/ml) at a concentration of 19 μg/ml. Cells were incubated on ice with: (a) Rh-EGF at 1.6×19^{-10} M (low occupancy experiments) or (b) Rh-EGF at 1.6×10^{-10} M plus unlabelled EGF at 10^{-6} M (low occupancy control) or (c) Rh-EGF at 2.4×10^{-7} M (high occupancy experiments). Incubations were always started on ice and no quantitative fluorescence measurements were made until at least 5 h had elapsed to allow a sufficiently close approach to equilibrium to be attained.

Dissociation of EGF from A431 cells

To demonstrate that, under conditions of low cell density and low temperature, EGF when present at 1.6×10^{-10} M is completely accessible to the extracellular medium, i.e., that no internalisation or sequestration occurs, a dissociation experiment was carried out. [^{125}I]EGF at 1.6×10^{-10} M was allowed to bind to A431 cells at low density ($\sim 10^3$ cells/cm^2 in 50 mm Petri dishes) for 6 h on ice after which unlabelled EGF at 5×10^{-8} M was added to each dish. Dissociation at 4°C was followed over a period of 5.25 h by aspirating the binding medium at each time point and washing the cells rapidly three times with ice-cold medium. Zero time was taken as that point after addition of unlabelled EGF followed by immediate washing.

Fluorescence microscopy and photobleaching recovery

Measurement of lateral diffusion by the fluorescent photobleaching recovery method was made with the instrument described by Garland (1981) and Johnson and Garland (1982), using the 514.5 nm argon ion laser line, a 40 x water immersion objective, a focussed laser spot radius ($1/e^2$) of 1.2 μm and analogue measurement of the fluorescent signal. Diffusion coefficients were calculated by the 3-point analysis of Axelrod *et al.* (1976). Measurements to compare the fluorescence from cells exposed to differing concentrations of Rh-EGF were made with the same apparatus used in a photon counting mode. The microscopic distribution of fluorescence in cells was observed by expanding the laser beam to cover the whole objective field, and viewing with the aid of a sensitive video camera (model 732 SIT camer, JAI a/s, Denmark). The cover slips on which cells had been grown were mounted for microscopy by one of two methods. Either the inverted cover slip was placed on a ring of wax surrounding a large drop of medium on a microscope slide, or the cover slip was assembled into a microscope observation chamber (model TCS1, Bachofer GmGM, 7410 Rentlingen, FRG). The latter method is preferable, and allows the medium around the cells to be changed. Temperature control of the sample on the microscope stage was achieved by mounting the slide upon the brass plate thermostatted with circulating water. The temperature of the sample was measured with a thermistor mounted on the slide.

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