Fluorescent-Labeled Growth Factor Molecules Serve as Probes for Receptor Binding and Endocytosis†

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ABSTRACT: In this report, we describe the applicability of fluorescein-labeled EGF (FITC-EGF) in monitoring the interaction between EGF and its cellular receptor in real time. This work takes advantage of previous studies that demonstrated that EGF may be labeled at its amino terminus with FITC without significant deleterious effects on the binding of the growth factor to its receptor or on the ability of the growth factor to activate the intrinsic tyrosine kinase activity of the receptor. When suspended human epidermoid carcinoma (A431) cells were treated with FITC-EGF, a biphasic quenching of the FITC fluorescence was observed. Both phases were blocked when the experiments were performed in the presence of excess unlabeled EGF. The first phase, in which the FITC emission was quenched by 8–10%, was complete within 30 s. This rapid quenching was attributed to changes in the rotational mobility of the EGF molecule that accompany its binding to receptors. The slower phase required 20–30 min for completion and resulted in the further quenching of the FITC fluorescence by 30–40%. This slower phase appeared to reflect the internalization of the receptor and its routing to acidic intracellular compartments. The rapid fluorescence decay phase was used to determine the rate constants (k_{on} and k_{off}) for the interaction of FITC-EGF with receptors on the surface of cells.

The addition of epidermal growth factor (EGF)[1] to cells containing its specific receptor (M_r ~170 000) results in a variety of cellular responses, including the stimulation of inositol lipid metabolism, changes in the cytosolic concentrations of a number of ions, receptor endocytosis, alterations in transcriptional regulation, and the stimulation of DNA synthesis and mitosis (Carpenter & Cohen, 1990). The mechanism by which the growth factor signal is propagated across the plasma membrane to induce many of these intracellular signals is still not clear, although different mechanistic models that involve either EGF-stimulated changes in the conformation of EGF receptor monomers (Koland & Cerione, 1988) or EGF-stimulated receptor dimerization (Boni-Schnetzler & Pilch, 1987; Schlessinger, 1986, 1988) have been proposed. In order to distinguish between the different proposed mechanisms for growth factor receptor activation, it will be necessary to compare the kinetics of growth factor binding to the time courses for receptor tyrosine kinase activation and the associated signaling events.

As a first step toward obtaining this kinetic information, we have examined the feasibility of a relatively straightforward fluorescence approach for monitoring directly the growth factor binding event in real time. In this study, we take advantage of two properties of the fluorescein isothiocyanate-labeled EGF molecule (FITC-EGF) that make it an excellent potential reporter group for growth factor/receptor interactions. One is the relatively short fluorescence lifetime of the probe (~2 ns). Proteins in the size range of the EGF molecule (~6 kDa) rotate in solution with a correlation time of 1–5 ns. Thus, the FITC-EGF molecule has time to tumble in solution before the fluorescence emission. It is therefore feasible to use changes in the rotational mobility of the growth factor molecule that accompany binding to the receptor as a direct monitor for the binding event.

The second property of the FITC-EGF molecule that provides an advantage for direct binding studies is the pH sensitivity of the spectroscopic properties of the FITC moiety. The protonation of the phenolic hydroxyl group of fluorescein (pK_a ~6.0) causes a distinct change in the absorption spectrum of the FITC which then results in a dramatic quenching of the fluorescein emission. This property can be used to monitor directly the endocytosis of FITC-EGF/receptor complexes and the routing of these complexes to low-pH intracellular compartments (pH 5–6; Sorkin et al., 1988). In the results presented below, we utilize these properties of the FITC-EGF molecule to assay growth factor binding to the EGF receptor, both in isolated plasma membranes from human epidermoid carcinoma (A431) cells and in suspended cells, as well as to read-out receptor endocytosis in real time.

MATERIALS AND METHODS

Reagents and Cells. FITC-EGF and EITC-EGF were prepared and handled as described previously (Carraway et al., 1989). A431 cells were grown in 100-mm dishes in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, and were suspended by Ca^{2+} depletion (Carraway & Cerione, 1991). Plasma membranes from A431 cells were purified as described previously (Carraway et al., 1989). Estimates for the number of binding sites used in each experiment were based on 2×10^6 [^{125}I]EGF binding sites per suspended cell (K. L. Carraway, unpublished observations),

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[1] Abbreviations: EGF, epidermal growth factor; FITC, fluorescein isothiocyanate; FITC-EGF, EGF labeled at its amino terminus with FITC; EITC-EGF, EGF labeled with eosin isothiocyanate; HEPES, *N*-2-(hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HBS, HEPES-buffered saline; PAO, phenylarsine oxide.

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and on the number of measured [^{125}I]EGF binding sites for a particular membrane preparation.

[^{125}I]EGF Binding by Suspended A431 Cells. A total of 8×10^6 cells were first suspended in 2 mL of HEPES-buffered saline (HBS: 20 mM HEPES/Na, pH 7.4, 0.14 M NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 1.8 mM $CaCl_2$, 0.35 g/L sodium bicarbonate, and 1 g/L D-glucose) or in HBS with no D-glucose, but supplemented with 10 mM 2-deoxyglucose and 10 mM NaN_3 (HBS/deGlu), at room temperature, and then binding measurements were initiated with the addition of 1 nM radiolabeled growth factor; 125-μL aliquots were removed at the indicated times and immediately filtered with HAWP filters (Millipore).

Steady-State Fluorescence and Anisotropy Measurements. Fluorescence measurements with intact cells were made using an SLM 8000 spectrofluorometer operated in the photon counting mode as described previously (Carraway et al., 1989). FITC-EGF was excited at 460 nm to minimize light scattering from cells. EITC-EGF was excited at 490 nm, and its emission was recorded at 544 nm. Steady-state anisotropy measurements were made with the fluorometer in the T configuration, with the emission monochromator set to 524 nm to obtain the horizontally polarized emission, and with a 524-nm bandpass filter to obtain the vertically polarized emission. Kinetic traces (such as that shown in Figure 5) for the vertically and horizontally polarized emission were obtained and stored independently. The background fluorescence from the cells was subtracted from the traces, and the fluorescence intensities were converted to anisotropy (A) with the equation:

$$A = [(I_V/I_H)/G - 1]/[(I_V/I_H)/G + 2] \quad (1)$$

where I_V/I_H is the ratio of the vertical and horizontal emission intensities when the excitation polarizer is in the vertical position and G is the same ratio when the excitation light is horizontally polarized.

The rotational correlation times (ϕ) for FITC-EGF free in solution and bound to membrane receptors were estimated from the Perrin equation:

$$A_0 = A(1 + kT\phi/V_h\eta) \quad (2)$$

where A_0 is the limiting anisotropy in the absence of all rotation, k is Boltzmann's constant, T is the temperature, V_h is the partial molecular volume of a hydrated sphere, and η is the viscosity. Perrin plots were constructed by measuring the anisotropy of FITC-EGF (either free in solution or bound to isolated plasma membranes from A431 cells) as a function of temperature over a range of 2–37 °C. The viscosity at each temperature was determined from standard tables (Weast, 1979).

Binding Data Analysis. In general, the overall anisotropy of a system of heterogeneous components is given by

$$A = \sum A_i f_i \quad (3)$$

where A is the observed anisotropy, A_i is the anisotropy of the ith component, and f_i is the mole fraction of the ith component. For the EGF receptor/FITC-EGF system, this may be simplified to

$$A = A_F f_F + A_B(1 - f_F) \quad (4)$$

where A_F and A_B are the anisotropies of the free and receptor-bound fluorescent growth factors, respectively, and f_F is the fraction of free FITC-EGF. A_F and A_B were determined to be 0.075 ± 0.005 and 0.18 ± 0.010, respectively, by measuring the anisotropy of 3 nM FITC-EGF in the presence of cells containing 50 nM EGF binding sites in the presence (A_F) and absence (A_B) of a 200-fold excess of unlabeled EGF. Since the dissociation constant for the FITC-EGF interaction with EGF receptors on the surface of A431 cells is 1.5 nM (Carraway & Cerione, 1991), ~97% of the FITC-EGF in solution should be bound to receptors under the conditions used to measure A_B.

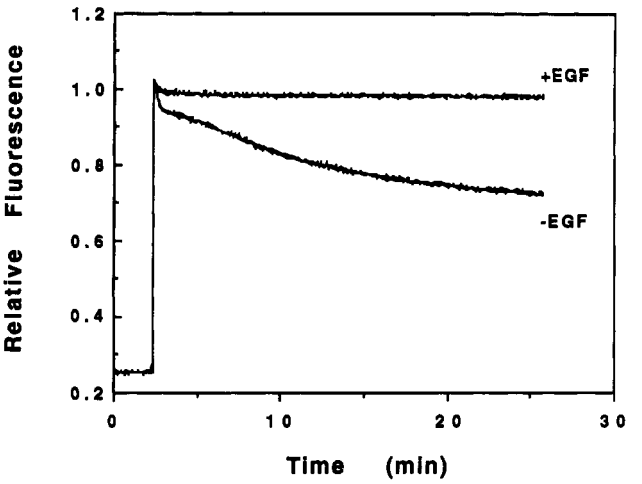

FIGURE 1: Quenching of FITC-EGF fluorescence after addition to suspended A431 cells. A431 cells were suspended in HBS to a density of 6×10^6 cells/mL (20 nM EGF receptor binding sites), stirred rapidly in a cuvette at ambient temperature, and treated with or without 440 nM EGF as indicated. An emission trace (excitation 460 nm, emission 518 nm) was started, and at time t = 2.5 min, FITC-EGF was added to 5 nM.

Binding and dissociation data were analyzed using a single-site model (see Discussion). The apparent rate constant for the dissociation of FITC-EGF from cell-surface receptors (k_{off}) was determined by fitting the anisotropy decay with time obtained when prebound FITC-EGF was competed from receptors with a 100-fold excess of unlabeled EGF to the equation:

$$A(t) = A_F + (A_N - A_F)[\exp(k_{off}t)] \quad (5)$$

where A_N is the anisotropy before the addition of unlabeled EGF and t is the time after its addition. The apparent rate constant for the association of FITC-EGF with cell receptors (k_{on}) was obtained by fitting the anistropy increase with time obtained after the addition of this derivative to cells to eq 4, where f_F as a function of time was derived previously [see Erickson et al., (1987)].

RESULTS

Interaction of FITC-EGF with A431 Cells. FITC-EGF undergoes biphasic changes in its steady-state fluorescence upon interaction with EGF receptors on the surface of A431 cells. Figure 1 shows the fluorescence at 518 nm as a function of time when 5 nM FITC-EGF was added to suspended A431 cells containing 20 nM [^{125}I]EGF binding sites at ambient temperature, in the presence and absence of an excess of unlabeled EGF. Immediately after addition of the fluorescent growth factor to the cells (within 1–3 s), there was a 4-fold increase in the fluorescence relative to the background emission observed with cells alone, and then a fraction of the fluorescence rapidly decayed over the next 30 s. Following this initial decay, there was a further slow decrease in fluorescence intensity over the next 20 min, which started to level off at ~25 min. Both phases of the fluorescence decay were blocked when cells were pretreated with an excess of unlabeled EGF,

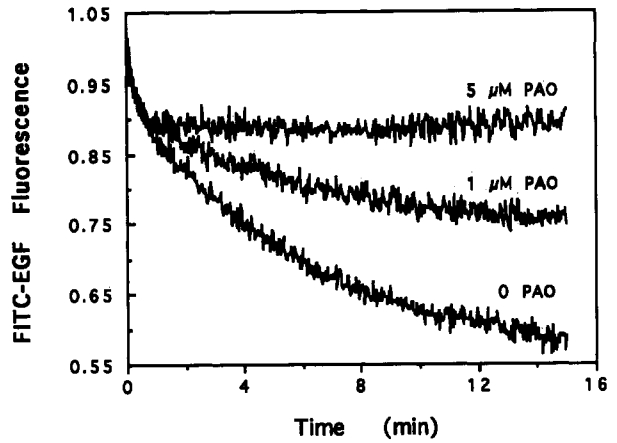

FIGURE 2: Inhibition of the slow fluorescence decay by PAO. A431 cells were suspended in HBS to a density of 6×10^6 cells/mL (20 nM sites), stirred rapidly in a cuvette, and treated with phenylarsine oxide at the indicated concentrations for 15 min at 37 °C. At time $t = 0$, FITC-EGF was added to 5 nM, and the fluorescence at 518 nm was followed with time.

suggesting that the fluorescence changes resulted from receptor-mediated events. The kinetics of both the rapid and the slower phases appeared to increase when the experiment was performed at 37 °C (see below). At 4 °C, the rapid phase was slowed considerably, while the slow fluorescence decay was blocked (not shown). When eosin isothiocyanate-labeled EGF (EITC-EGF) was added to suspended A431 cells at room temperature and 37 °C, only the slow phase of the fluorescence decay was observed (data not shown). Because EITC-EGF interacts with the EGF receptor with an affinity similar to that of FITC-EGF (Carraway et al., 1989; Carraway & Cerione, 1991), the absence of the rapid fluorescence decay is probably due to its shorter fluorescence lifetime (discussed below).

The Slow Phase of the Fluorescence Decay Correlates with Endocytosis. The time course of the slow phase of the fluorescence decay of FITC-EGF suggested that it was due to a process that occurred well after the initial binding of the growth factor to the receptor. A number of previous studies have demonstrated that EGF binding stimulates the uptake of its receptors from the surface of most cells, and induces their subsequent down-regulation through degradation in intracellular lysosomal compartments [see Carpenter (1987)]. Our previous results showed that the time course for [^{125}I]-EGF uptake from the surface of suspended A431 cells paralleled the time course for the slow fluorescence change for FITC-EGF and EITC-EGF after addition of these derivatives to the suspended cells (Carraway & Cerione, 1989). Moreover, when the metabolic inhibitors deoxyglucose and sodium azide were present in the suspension medium, both [^{125}I]EGF uptake and the slow phase of fluorescence quenching were blocked, suggesting that the cellular process responsible for this phenomenon required an energy input.

To further determine whether receptor endocytosis might be responsible for the slow phase of the fluorescence decay, we examined the effect of a more specific endocytosis inhibitor on the time courses for the slow fluorescence change. Phenylarsine oxide (PAO) has been shown to potently and specifically inhibit EGF receptor endocytosis (Walker & Burgess, 1988), possibly as an outcome of an inhibition of protein phosphatase activity. Figure 2 shows the time course for the changes in the FITC-EGF fluorescence in A431 cells in the absence of PAO and in the presence of 1 and 5 μM PAO. In the absence of the endocytosis inhibitor, there was

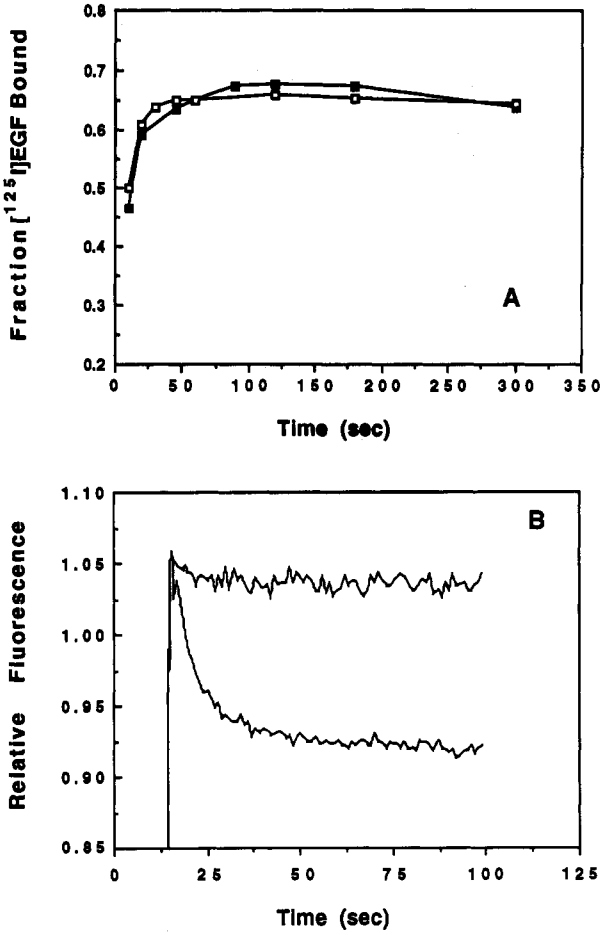

FIGURE 3: Comparison of [^{125}I]EGF binding and the rapid FITC-EGF fluorescence decay. (A) A431 cells were suspended in either HBS (■) or HBS/deGlu (□) to a density of 6×10^6 cells/mL (20 nM sites) and stirred rapidly in a cuvette at ambient temperature. At time $t = 0$, [^{125}I]EGF was added to 1 nM, and at the indicated times, aliquots were removed and filtered to separate bound from free [^{125}I]EGF. Plotted is the fraction of the total added [^{125}I]EGF that associated with cell-surface receptors. (B) A431 cells were suspended to a density of 6×10^6 cells/mL in HBS, and treated without (lower trace) or with (upper trace) 440 nM EGF. 5 nM FITC-EGF was added at time $t = 15$ s, and the fluorescence (excitation 460 nm, emission 518 nm) was followed with time.

a steady decline in the FITC-EGF fluorescence (40–50% quenching) over a time period of 10–20 min at 37 °C. However, pretreatment of cells for 15 min with 1 μM PAO partially blocked the fluorescence quenching observed after ~1 min, while 5 μM PAO completely inhibited the slower phase of FITC-EGF fluorescence quenching. It should be noted, however, that the presence of PAO had no detectable effect on the rapid phase of quenching.

Together with our previous observations, these data suggest that the slower fluorescence changes that accompany the interaction of the labeled growth factors with suspended cells reflect at least some aspect of the endocytic process. The low pH of the endosomes and lysosomes is probably responsible for quenching the fluorescence of the labeled EGF molecules. The lower extent of fluorescence quenching generally observed with the eosin derivative of EGF (data not shown) is consistent with its higher pK_a relative to that of fluorescein.

The Rapid Phase of the Fluorescence Decay Correlates with FITC-EGF Binding to Receptors. Figure 3 shows a comparison of the specific binding of 1 nM [^{125}I]EGF to suspended A431 cells containing 20 nM binding sites with the rapid phase of the fluorescence decay observed upon addition of 5 nM FITC-EGF to suspended cells under identical

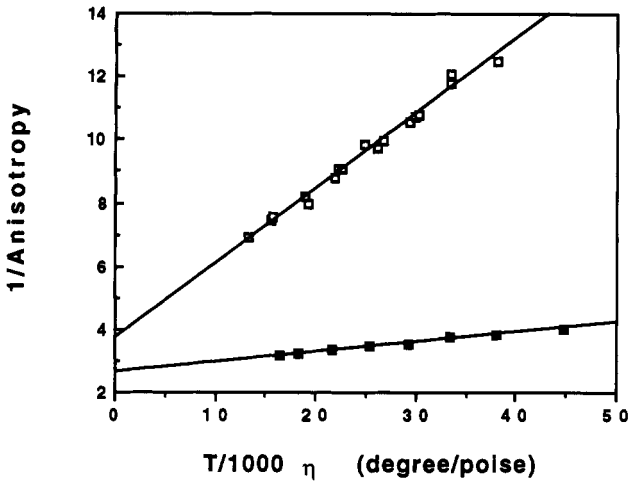

FIGURE 4: Perrin plot of receptor-bound and free FITC-EGF. The anisotropy (excitation 460 nm, emission 524 nm) of 5 nM FITC-EGF free in solution (□) or bound to A431 plasma membranes containing 20 nM [^{125}I]EGF binding sites (■) was determined as outlined under Materials and Methods. Different values for T/η were obtained by varying the temperature from 2 to 37 °C.

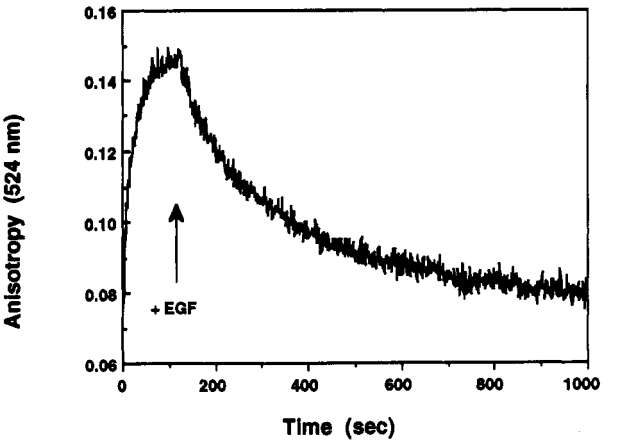

FIGURE 5: Binding of FITC-EGF to EGF receptors in A431 cells determined by changes in anisotropy. A431 cells were suspended in HBS/deGlu to a density of 6×10^6 cells/mL (20 nM sites) and stirred rapidly in a cuvette at 4 °C. At time $t = 0$ s, FITC-EGF was added to 5 nM, and the anisotropy (excitation 460 nm, emission 524 nm) was followed with time. At time $t = 115$ s, EGF was added to 440 nM.

conditions. The binding of [^{125}I]EGF was very rapid; the reaction was ~75% completed before the first time point could be collected at 15 s, and was completely finished by 30 s. The time course for the initial fluorescence decay was similar, and was blocked by an excess of unlabeled EGF. These data strongly suggest that this initial fluorescence decay results from the binding of the fluorescent growth factor to the receptor, and point to the possibility that this fluorescence change may be used to analyze the kinetics of the interaction of FITC-EGF with the EGF receptor.

The Rapid Phase of the Fluorescence Decay Results from Increased Polarization of the Growth Factor upon Binding to Its Receptor. EGF is a very small (M_r = 6000), roughly spherical molecule (Cohen, 1962; Carpenter & Cohen, 1979) which tumbles rapidly in three dimensions when free in solution. Upon binding to the EGF receptor in membranes, the rotational mobility of the growth factor is dramatically reduced because of the much higher molecular weight of the complex ($M_r \geq 175\,000$) and the fact that the lipid bilayer restricts the rotational mobility to one dimension. Hence, if EGF were labeled with a fluorescent probe that has a fluorescence lifetime similar to the rotational correlation time of the growth factor, a significant enhancement in the fluorescence anisotropy of this probe might be observed upon binding of the labeled growth factor to membrane receptors.

Figure 4 shows Perrin plots for FITC-EGF free in solution and bound to plasma membranes from A431 cells. This method of analysis (based on eq 2) allows for the comparison of the rotational mobilities of the labeled growth factor under various conditions. The y intercepts of these plots represent A_0^{-1}, the limiting anisotropy in the absence of all rotational mobility, and are similar whether FITC-EGF is free in solution or bound to membrane receptors. (The small difference in the values for A_0^{-1} for bound and free FITC-EGF probably results from the greater turbidity of the sample containing membranes.) The slopes of these plots give a measure of the extent of rotational freedom of the fluorescent probe under the two conditions; the steeper slope for the case of free (unbound) FITC-EGF reflects a significantly greater rotational mobility of the growth factor under these conditions. From eq 2, the rotational correlation times of the free and receptor-bound fluorescein-labeled growth factor were estimated to be 1.09 and 2.15 ns, respectively. Sedimentation studies with unlabeled EGF suggest that a rotational correlation time of 2.1–2.5 ns should be expected for the growth factor free in solution [see Carpenter and Cohen (1979)]. Hence, it appears that the fluorescein moiety on the EGF molecule has a significant degree of rotational freedom independent of that of the growth factor, but is still sensitive to the differences in the rotational mobility of the EGF molecule upon binding to its receptor.

In the experiment shown in Figure 1, no attempt was made to correct for the polarizing effects of the emission monochromator; neither the excitation nor the emission polarizers were in place when the fluorescence traces were obtained. However, when the same experiment was performed under conditions where the excitation polarizer was set to 0° and the emission polarizer was set to 55.3°, the rapid fluorescence decay response was eliminated (not shown). Under these "magic angle" conditions (Lakowicz, 1983), the polarizing effects of the emission monochromator are corrected such that the detected emission is not altered by changes in the rotational mobility of the fluorophore. Hence, it appears that the rapid phase of the fluorescence decay correlates with FITC-EGF binding to the EGF receptor, and reflects the accompanying changes in the rotational mobility of the growth factor upon binding. The lack of a fluorescence change observed with EITC-EGF upon binding to the receptor is consistent with the very short lifetime for eosin fluorescence ($\tau \sim 0.2$ ns). Such lifetimes are too short to be sensitive to changes in the rotational mobility of EGF.

Analysis of the Kinetics of FITC-EGF Binding to the EGF Receptor on Suspended A431 Cells. The observation that the polarization of FITC-EGF changes in response to binding suggests that this property may be used to measure the kinetics of FITC-EGF interaction with receptors on suspended cells and in membranes in real time. Figure 5 shows that the anisotropy of FITC-EGF increased roughly 2-fold upon binding to receptors on the surface of suspended A431 cells. Figure 6 shows that a similar increase was observed when FITC-EGF was added to purified plasma membranes. The anisotropy increase occurred with a time course consistent with that of the fluorescence quenching shown in Figure 3B, and was blocked when cells were pretreated with an excess of unlabeled EGF (Figure 6). Figure 5 shows that the anisotropy change was completely reversible. The addition of a 100-fold

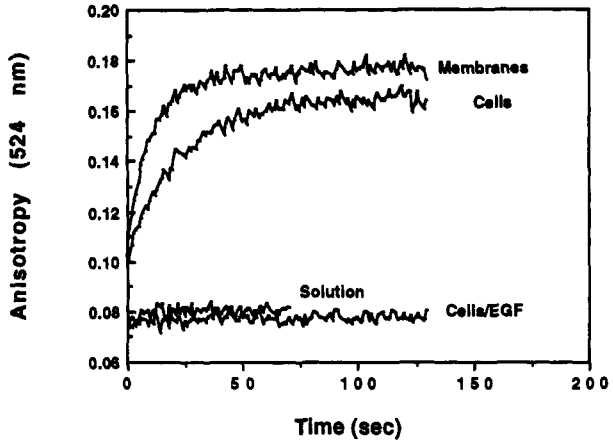

FIGURE 6: Comparison of FITC-EGF anisotropy changes in cells and membranes. A431 cells suspended to a density of 6×10^6 cells/mL (20 nM receptor sites) in HBS/deGlu (treated without and with 440 nM EGF, as indicated) or plasma membranes containing 20 nM [^{125}I]EGF binding sites were stirred rapidly in a cuvette at ambient temperature. At time $t = 0$, FITC-EGF was added to 5 nM, and the anisotropy (excitation 460 nm, emission 524 nm) was followed with time.

excess of unlabeled EGF caused the anisotropy to drop to its initial level over a period of time consistent with the release of the fluorescent growth factor from cell-surface receptors (Wiley, 1988).

Using the general procedure for the experiment described for Figure 5, that is, recording the changes in the fluorescence anisotropy of FITC-EGF after addition of this derivative to suspended A431 cells and the subsequent displacement by an excess of unlabeled EGF, the rate constants for binding and displacement of EGF from its receptor in intact cells were obtained under a variety of conditions. These experiments were performed in the HBS/deGlu buffer to prevent the acid quenching of FITC-EGF fluorescence that accompanies endocytosis. Anisotropy traces such as those depicted were fit to eq 4 and 5 to obtain the values for k_{on} and k_{off}, the apparent rate constants for the FITC-EGF/EGF receptor interaction. Figure 7 shows the effect of temperature on these constants when FITC-EGF interacted with receptors on the surface of suspended A431 cells. Interestingly, as the temperature was increased from 2 to 37 °C (275–310 K), the k_{off}/k_{on} ratio remained constant at 0.0045 μM, indicating that the equilibrium constant for the FITC-EGF/receptor interaction remained constant over a significant temperature range. These observations are in agreement with equilibrium binding data with [^{125}I]EGF which indicate that the dissociation constant of the ligand–receptor interaction is roughly constant whether the experiment is performed at 0, 23 or 37 °C (not shown). These data suggest then that the contribution of entropy to the interaction of EGF with its receptor predominates over any enthalpic contributions, perhaps emphasizing the role of hydrophobic bonds in this interaction.

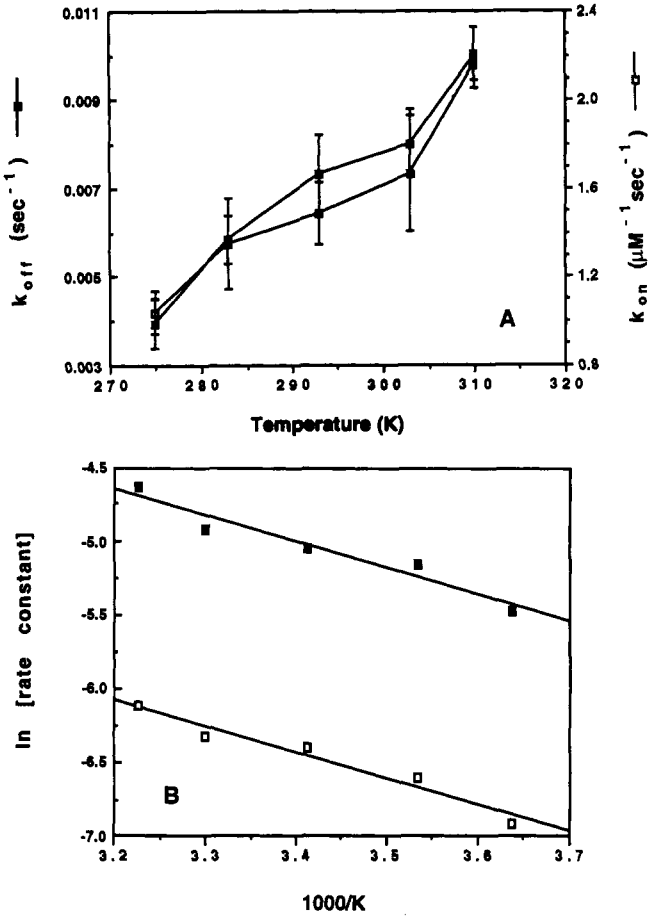

FIGURE 7: Effect of temperature on the forward and reverse rate constants for FITC-EGF interaction with EGF receptors in suspended A431 cells. A431 cells were suspended to a density of 1.8×10^7 cells/mL (60 nM sites) in HBS/deGlu, and equilibrated to the indicated temperatures by rapid stirring in the thermostated sample chamber. Kinetic traces for the binding and displacement of FITC-EGF were obtained as described for Figure 5, and the forward and reverse rate constants were obtained from eq 4 and 5, respectively. (A) Depicted are the average values and the errors obtained from three determinations of the forward (□) and reverse (■) rate constants at each temperature. (B) Arrhenius plot of the average values for k_{on} (□, ln nM^{-1} s^{-1}) and k_{off} (■, ln s^{-1}) depicted in panel A, emphasizing the parallel increase in the rate constants as a function of temperature.

DISCUSSION

Two of the initial responses to EGF stimulation of cells are the activation of the intrinsic protein tyrosine kinase activity of the EGF receptor and the internalization and down-regulation of ligand-occupied receptors. In response to growth factor binding, the tyrosine kinase activity is immediately activated, and receptors which are initially randomly dispersed on the cell surface become clustered. Clustering has been visualized by fluorescence microscopic (Haigler et al., 1978; Schlessinger et al., 1978) and electron microscopic techniques (Van Belzen et al., 1988). The clustering of EGF receptors occurs within 2–10 min of growth factor binding and is due to the co-localization of tens to perhaps hundreds of receptors in clathrin-coated pits at the cell surface. Over the following 20–40 min, clathrin-coated pits are internalized, and the majority of the EGF/receptor complexes are delivered to endosomes and lysosomes for processing. EGF remains bound to its receptor throughout most of this intracellular routing (Carpentier et al., 1987). The entire process of EGF-stimulated receptor down-regulation and processing, including the co-localization of EGF receptors in coated pits, can be inhibited at low temperatures (≤10 °C), even though EGF-stimulated receptor autophosphorylation and phosphorylation of cellular substrates are not inhibited under these conditions (Carpentier et al, 1987). The presence of metabolic inhibitors in the medium prevents the uptake of receptors into coated pits (Schlessinger et al., 1978).

In an effort to better understand the mechanistic details of EGF receptor response to growth factor stimulation, we have been developing spectroscopic methods for examining EGF/receptor and receptor/receptor interactions (Carraway et al., 1989; Carraway & Cerione, 1990, 1991). Fluorescence spectroscopy offers a number of advantages in examining the roles of protein–ligand and protein–protein interactions in

receptor-coupled signal transduction pathways (Phillips & Cerione, 1988; Carraway et al., 1989; Erickson & Cerione, 1991). In particular, fluorescence approaches allow such interactions to be observed in real time, and allow the collection of many more data points than can be obtained from radiolabeled ligand techniques. Our previous studies have focused on the use of resonance energy transfer, a fluorescence spectroscopic technique, to examine the role of receptor–receptor interactions in signal transduction by the EGF receptor. One question that has arisen concerns how the rates of EGF-stimulated receptor dimerization compare with those for growth factor binding, receptor tyrosine kinase activation, and receptor internalization. It has therefore become necessary to develop spectroscopic methods to examine the kinetics of these events. In the present study, we demonstrate that EGF molecules labeled at their amino termini with fluorescent isothiocyanate derivatives serve as excellent reporter groups for the binding of the growth factor molecule to its cell-surface receptor, and the subsequent internalization of the ligand/receptor complex.

The binding of FITC-EGF to the EGF receptor on the surface of A431 cells results in a quenching of the emission of the fluorophore, which stems from a significant change in the rotational mobility of the fluorophore upon ligand binding. The more restricted mobility of the receptor-bound FITC-EGF yields a more polarized fluorescence emission, which when measured in the absence of polarizers yields an apparent quenching of the fluorophore fluorescence. The ability to monitor the binding of FITC-EGF offers a significant advantage over the use of [^{125}I]EGF to measure the kinetics of EGF binding because many more fluorescence points may be collected over the course of the binding reaction. It should also be noted that the FITC-EGF derivative is fully functional relative to the unlabeled EGF molecule, in its abilities to stimulate receptor–receptor interactions and receptor tyrosine kinase activity *in vitro* (Carraway et al., 1989), and elicit events in cells such as changes in the intracellular [Ca^{2+}] (Carraway & Cerione, 1991) and the stimulation of cell growth (K. L. Carraway, unpublished data).

From the fluorescence traces obtained in these studies, we have derived rate constants for FITC-EGF association (k_{on}) with and dissociation (k_{off}) from EGF receptors on the surface of A431 cells. It should be emphasized that these values represent apparent rate constants as determined by this experimental system, rather than the rates intrinsic to the binding interaction. In our analysis, we assume that the fluorescent growth factors bind to a single class of receptors in the A431 cells. While equilibrium binding studies with [^{125}I]EGF reveal a single class of EGF receptors with a dissociation constant of 1 nM on the surface of the suspended cells (unpublished results), a number of studies with adherent cells suggest multiple classes of sites with different affinities for the radiolabeled ligand (Kawamoto et al., 1983; Defize et al., 1989; Bellot et al., 1990). Hence, the apparent rate constants may reflect interactions with more than one receptor class. In addition, heterogeneity in the samples (for example, the presence of poorly stirred layers of cells within the cuvette) might also contribute to deviations between the intrinsic rate constants and those measured with this experimental system. However, the values that we have derived for k_{on} [$(1–2) \times 10^6$ M^{-1} s^{-1}] and k_{off} [$(4–10) \times 10^{-3}$ s^{-1}] compare favorably with ranges of values reported previously for [^{125}I]EGF interaction with EGF receptors [$(2–5) \times 10^6$ M^{-1} s^{-1} and $(2–50) \times 10^{-3}$ s^{-1} for k_{on} and k_{off}, respectively; Knauer et al., 1984; Myers et al., 1987; Waters et al., 1991].

The slower phase of the quenching of the FITC-EGF fluorescence is an outcome of the pH-sensitivity of the FITC moiety. The incorporation of the FITC-EGF/receptor complex into low-pH endosomes results in a significant quenching of the fluorophore emission. Like the case for the initial growth factor binding event, this offers a significant advantage for measuring accurately the kinetics of receptor endocytosis under different conditions (for example, in the presence of suspected endocytosis inhibitors or regulators) because of the ease in obtaining numerous data points.

The ultimate utility of fluorescence anisotropy in examining EGF/receptor interactions will be in the elucidation of the physical mechanism by which the EGF receptor tyrosine kinase activity is activated upon ligand binding, and the role of receptor–receptor interactions in this activation event. Recent evidence suggests that receptor dimerization may represent a rate-limiting step in the activation of the tyrosine kinase activity of detergent-solubilized EGF receptor (Canals, 1992). However, it appears that the inhibition of receptor–receptor interactions in plasma membranes by a specific monoclonal antibody has no impact on EGF-stimulated tyrosine kinase activity (Carraway & Cerione, 1993). These observations suggest that there might be a difference in the mechanism by which EGF activates its receptor in membranes and in a detergent solution. The use of fluorescence will facilitate comparisons of the rates of EGF binding with the rates for receptor dimerization in membranes. Both of these events may be assessed by fluorescence spectroscopic methods. It should be possible to compare directly the rates of these processes with the kinetics of receptor-catalyzed *in vitro* phosphorylation of tyrosine-containing substrates (peptide substrates and known protein substrates such as phospholipase C-γ). Only through careful, detailed comparisons of the rates of EGF binding, aggregation, and tyrsoine kinase activation will it be possible to determine the relationship between these receptor events.

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