The Role of Tyrosine Kinase Activity in Endocytosis, Compartmentation, and Down-regulation of the Epidermal Growth Factor Receptor*

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Occupancy-induced down-regulation of cell surface epidermal growth factor (EGF) receptors attenuates signal transduction. To define mechanisms through which down-regulation of this class of growth factor receptors occurs, we have investigated the relative roles of ligand-induced internalization and recycling in this process. Occupied, kinase-active EGF receptors were internalized through a high affinity, saturable endocytic system at rates up to 10-fold faster than empty receptors. In contrast, full length EGF receptors lacking tyrosine kinase activity underwent internalization at a rate independent of occupancy. This "kinase-independent" internalization rate appeared to reflect constitutive receptor internalization since it was similar to the internalization rate of both receptors lacking a cytoplasmic domain and of antibodies bound to empty receptors. EGF internalized by either kinase-active or kinase-inactive receptors was efficiently recycled and was found within endosomes containing recycling transferrin receptors. However, targeting of internalized receptors to lysosomes did not require receptor kinase activity. All receptors that displayed ligand-induced internalization also underwent down-regulation, indicating that the proximal cause of down-regulation is occupancy-induced endocytosis. Tyrosine kinase activity greatly enhances this process by stabilizing receptor association with the endocytic apparatus.

The cell surface is a dynamic structure in which specific components are continually added and removed. Among the most extensively regulated cell surface components are receptors because they constitute the primary means by which cells perceive their environment. Although significant progress has been made in defining the mechanisms that regulate the internalization and sorting of receptors that bind metabolically important ligands such as transferrin (Tf)1 and low density lipoprotein (LDL), the mechanisms involved in down-regulation of signal transducing receptors with intrinsic tyrosine kinase activity remain an important unsolved question in cell biology.

The Tf and LDL receptors, whose primary function is bulk transport of nutritional molecules into the cell (Kaplan, 1981), cluster about coated pits which constitute approximately 2% of the cell surface (Anderson et al., 1977). Internalization and trafficking of Tf and LDL receptors is independent of ligand binding because clustering in coated pits (Beisiegel et al., 1981; Watts, 1985), receptor internalization, and subsequent recycling (Ajoka and Kaplan, 1986; Basu et al., 1981; Stein and Sussman, 1986) is observed in both the presence and absence of ligand. Because the behavior of both occupied and unoccupied receptors is similar, ligand binding does not normally alter surface receptor number or their intracellular distribution. In the case of LDL receptors, ligand dissociates in the acidic environment of the endosomes and is targeted to lysosomes while empty receptors recycle back to the cell surface (Brown et al., 1983). In the case of Tf receptors, ligand remains with the receptor and both recycle back to the cell surface (Klausner et al., 1983).

In contrast to nutrient receptors, signaling receptors undergo ligand-induced conformational changes that alter their endogenous enzymatic activity and distribution. Empty EGF receptors are randomly distributed at the cell surface (Willingham et al., 1983), but upon binding ligand they cluster in coated pits and are internalized (Haigler et al., 1979). Ligand binding also leads to activation of receptor tyrosine kinase activity and a decrease in surface receptor number (down-regulation). Down-regulation of EGF receptors attenuates signal transduction (Chen et al., 1989; Wells et al., 1990), and requires receptor kinase activity, but the mechanistic basis of the kinase requirement is controversial (Glenney et al., 1988; Honegger et al., 1987a). We have proposed that in the case of full length EGF receptor, activation of its intrinsic tyrosine kinase activity leads to high affinity binding to coated pits (Chen et al., 1989; Glenney et al., 1988; Lund et al., 1990b). The resulting increase in internalization rate shifts receptors to an intracellular compartment which is then targeted to lysosomes (Stoscheck and Carpenter, 1984; Wiley,

The abbreviations used are: Tf, transferrin; IS-, intracellular sequence negative; LDL, low density lipoprotein; PVP, polyvinylpyrrolidone; k, specific endocytic rate constant; k, recycling rate constant; k, ligand degradation rate constant; EGF, epidermal growth factor; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HRP, horseradish peroxidase; DAB, diaminobenzidine.

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1985). However, other investigators have reported that ligand-induced internalization of the EGF receptor is independent of tyrosine kinase activity and that down-regulation is due to a kinase-mediated inhibition of EGF receptor recycling (Felder et al., 1990; Honegger et al., 1987a, 1990). A third possibility is that receptor kinase activity is involved in both endocytosis and postendocytic compartment. Distinguishing between these alternate possibilities is important because little is known regarding the mechanisms of ligand-induced endocytosis or the processes that regulate postendocytic sorting and targeting. In addition, each would require that an entirely different strategy be taken to determine the molecules involved in ligand-dependent down-regulation.

To accurately determine the rate of ligand-induced endocytosis, several factors must be considered. Unless a receptor is specifically excluded from a coated pit, it should always be subject to random entrapment (Goldstein et al., 1984). Thus, simply observing a finite endocytic rate for a hormone receptor does not prove that the process is ligand-induced. In addition, receptor-mediated endocytosis is a second order process: receptors compete for coated pits or other endocytic structures (Pearse, 1988; Wiley, 1988; Lund et al., 1990b). Thus the extent of receptor occupancy influences the fraction of occupied receptors entering the coated pit pathway. Once the endocytic components are saturated, receptor internalization can only proceed through a nonspecific mechanism. The effect of ligand recycling and degradation must also be considered. If these processes are fast relative to endocytosis, then estimates of ligand internalization rates will be too low (Wiley and Cunningham, 1982; Waters et al., 1990).

In the present study, we have examined the relationship between ligand-induced internalization, recycling, and down-regulation of the EGF receptor. We have also determined the influence of receptor tyrosine kinase activity on these processes. Using labeled monoclonal antibodies to follow unoccupied receptors, we found that empty receptors undergo endocytosis at the same rate as receptors lacking the cytoplasmic domain, indicative of random entrapment. Occupancy of EGF receptors lacking kinase activity did not change their internalization rate. However, occupancy of kinase-active receptors increased their internalization rate up to 10-fold. Both types of receptor recycled back to the cell surface at similar rates. Truncated, kinase-inactive receptors could undergo accelerated degradation when internalized due to ligand-induced endocytosis, indicating that tyrosine kinase activity is not required for lysosomal targeting. We conclude that ligand-induced EGF receptor down-regulation is mediated through alterations in endocytotic rather than recycling rates.

**EXPERIMENTAL PROCEDURES**

**General—**Mouse EGF was purified from submaxillary glands (Savage and Cohen, 1972). EGF, human, iron-loaded diphyc T-cell (Calbi-Ohm-Behring Corp.), and monoclonal antihuman EGF receptor antibody 528 IgG (Gill et al., 1984) were iodinated with either 125I or 111In (Amersham Corp.) using IODO-BEADS (Fierce Chemical Co.) according to the manufacturer's recommendations and free iodine separated from the radiolabeled ligands by dialysis or by passing the mixture over a 0.8 × 20-cm column of Sephadex G-10 equilibrated with PBS. The specific activity of 125I-labeled EGF was generally between 600 000 cpm/mmol and 1 000 000 cpm/mmol, 111In-labeled Tr was between 780 and 3100 cpm/mmol, and 125I-labeled 528 monoclonal was between 1300 and 1900 cpm/mmol. The 125I-EGF was between 240 and 430 cpm/ mmol. The fluid phase marker, [3H]polyvinylpyrrolidone ([3H]PVP) was synthesized as previously described (McKinley and Wiley, 1988).

**Cell Culture—**B2B mouse L cells, which contain no endogenous EGF receptors, and B2B cells transfected with normal (WT) or mutated (M121, c’647, c’973, c’1022, and M121c’1022) human EGF receptor were generated as previously described (Chen et al., 1989).

A modified dihydrofolate reductase gene was the selectable marker for all transfections. B2B cells were grown in Dulbecco's modified Eagle's medium (Flow Laboratories) containing dialyzed 10% calf serum (HyClone). 5 μM methotrexate was added to the medium for those cells transfected with human EGF receptor.

Expression studies—Cells grown to confluence in 35-mm dishes were switched from growth medium to serum free Dulbecco's modified Eagle's medium containing 20 mM HEPES (pH 7.4) and 0.1% bovine serum albumin and no bicarbonate (DHB) 1 h before experiments. Binding experiments were initiated by changing to DHB containing the indicated concentrations of labeled ligand. The addition of ligand and the rinsing of cells were done with a semiautomatic apparatus (Wiley and Cunningham, 1982). The temperature limits for binding experiments were 35.5–37°C, and the temporal resolution was within 10 s. Binding was terminated by rapidly rinsing six times with 2 ml of ice-cold WHIPS buffer (20 mM HEPES (pH 7.4), 130 mM NaCl, 5 mM KC1, 0.5 mM MgCl2, 1 mM CaCl2, 1 mg/ml polyvinylpyrrolidone). The relative amounts of ligand associated with the surface and interior of the cells was determined by acid stripping at 0°C using 50 mM glycine-HCl, 100 mM NaCl, 2 mg/ml PVP, 2 mM urea (pH 3.0). For pulse-chase studies in which viability of the cells after stripping was important, the stripping solution omitted the urea. Stripping efficiencies were determined in parallel and were generally 98% for the surface solutions and 90% in the absence of urea. Nonspecific binding was determined in the presence of at least 200-fold molar excess unlabeled ligand in the case of Tf, or by measuring binding to B2B cells that lack EGF receptors in the case of labeled EGF and 52B monoclonal antibody. Nonspecific binding was generally less than 5% of total binding. Single label experiments using 125I-labeled 528 IgG in the absence and presence of EGF established that EGF did not affect the specific internalization rate of the antibody. Cell number was determined with a Coulter Counter.

**Fluid Uptake Studies—**Cells were treated as described above for binding studies, except that 111InPVP was used. Cells were changed at 37°C to the indicated concentration of [111In]PVP and at appropriate times the medium was aspirated and the cells rinsed 10 times at 0°C with WHIPS solution. Cells were then removed from their plates with trypsinization and collected by centrifugation (McKinley and Wiley, 1988). The amount of fluid incorporation was corrected for cell number.

**Down-regulation—**To evaluate the ability of 528 monoclonal IgG to down-regulate surface receptor expression, cells were incubated with 13 nM 528 IgG in standard binding medium at 37°C. At appropriate times, cells were removed from the plates by scraping and dispersed by trituration. After centrifugation, aliquots of approximately 106 cells were resuspended in 50 μl of 528 monoclonal IgG at a concentration of 80 μg/ml. After 30 min on ice, cells were washed twice with PBS and then fluorescein isothiocyanate-conjugated antimoise IgG (50 μl of 20 μg/ml) was added for another 5 min of incubation with PBS and fixing in 1% glutaraldehyde in PBS. 5000 cells per sample were analyzed using a Becton-Dickinson fluorescence-activated cell sorter with FACS software. Mean fluorescence channels as the measurement of fluorescence intensity were converted to a linear scale between 1 and 104.

**Degradation of EGF—**Cells were incubated at 37°C with 0.5 mM 125I-EGF in DHB binding medium using a bovine serum albumin concentration of 100 μg/ml. At appropriate intervals, 100 μl samples were collected from the medium and loaded on 12.5% native tube polyacrylamide gels running at 4°C (Wiley and Cunningham, 1982). Free iodine, iodotyrosine, and EGF were cleanly separated in the gels during electrophoresis. Glycogen was cut and incubated with EGF quantified in the iodotyrosine peak. No free iodine was generated during incubation of cells with EGF. In addition, no degradation was observed when EGF was incubated with parental B2B cells which lack endogenous EGF receptors.

**Gradient Fractionation of Cells—**Subconfluent monolayers of cells grown in 100-mm plates were incubated with radiolabeled ligand at the indicated times at 37°C. After rinsing, surface-associated ligand was removed with 100 mM NaCl, 50 mM glycine (pH 3.0) for 2 min

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followed by rinsing with saline and 100 μM phenylarsine oxide for 10 min at 0 °C to render the cells fragile. Cells were removed from their plates by incubation with 600 μg/ml trypsin at 0 °C followed by neutralization with 5-fold excess soybean trypsin inhibitor. Cells were pelleted and homogenized in 1 ml of 250 mM sucrose, 10 mM triethanolamine, 1 mM EDTA (pH 7.4), by eight passages through a 25-gauge needle. Cell breakage was generally 80–90% as assayed by microscopy. Nuclei were removed by centrifugation at 900 × g for 5 min. Samples were loaded on linear 19–40% (w/w) sucrose/PVP gradients and brought to isoponic equilibrium by centrifugation in an SW 40 rotor at 37,500 rpm for 2 h at 4 °C (Opresko et al., 1980). Gradients were pumped out of the top of the tubes using an ISCO model 185 density gradient fractionator at a rate of 1 ml/min. Fractions of 0.3 ml were either counted or evaluated for enzymatic activity. Samples containing both 125I and 35S were counted on a three-channel Packard gamma spectrophotometer. All counts were corrected for channel spillover and counting efficiency. Sedimentation position of the plasma membrane was determined by binding of 125I-labeled wheat germ agglutinin or by surface iodination using sulfo-SHPP (Pierce Chemical Co.) at 0 °C. Both methods yielded identical results. Endosomes were identified by incubating cells to a steady state with 125I-Tf followed by removal of the surface-associated ligand with glycine-HCl prior to cell fractionation. Lysosomes were identified by hexosaminidase activity (Horvat et al., 1969) and mitochondria by cytochrome c oxidase activity (Sottocasa et al., 1967). Density profiles were determined by refractive index.

**Density Shifting of Endosomes**—Colocalization of different ligands with endosomes was established by the horseradish peroxidase (HRP)-diaminobenzidine (DAB) density shift technique (Courtoy et al., 1984; Ajikie and Kaplan, 1987). The conjugate between Tf and HRP (HRP-Tf) was a gift of Dr. Jerry Kaplan. A 1:1 conjugate of EGF and HRP (HRP-EGF) was prepared by the method of Nakane and Kawaoi (1974). The HRP was purified by CM-cellulose chromatography (Shannon et al., 1966) followed by conjugation to EGF. The HRP-EGF was isolated by sequential chromatography on P-100 (Bio-Rad) and CM-cellulose using a 0.05–0.1 M sodium acetate gradient (pH 4.4). The peak eluting at 0.06 M acetate was confirmed to be a 1:1 EGF-HRP conjugate by absorbance ratio at 280 and 400 nm as well as enzymatic activity and ability to compete with 125I-EGF for receptor binding. Cells in 100-mm dishes were incubated with 50 nM of either HRP-Tf or HRP-EGF and 50 nM of either 125I-Tf or 125I-EGF for 60 min at 0 °C followed by a shift to 37 °C for 15 min. Surface-associated ligand was removed by acid stripping and the cells were homogenized using 10 passages through a ball bearing homogenizer (Balch and Rothman, 1985). The postnuclear supernatants were treated with DAB and H2O2 (Ajikie and Kaplan, 1987) and then fractionated by isoponic sucrose gradients as described above.

**Receptor Degradation**—Cells expressing the indicated mutant EGF receptors were treated with or without 50 nM EGF for 24 h at 37 °C. Duplicate plates of cells were harvested using hot Laemml sample buffer, pooled and resolved on 7.5% sodium dodecyl sulfate-polyacrylamide gels, transferred to Immobilon membranes, and EGF receptors were detected using either monoclonal antibody C13 or polyclonal antibody to both down-regulate and to internalize EGF. As shown in Fig. 1, the 15E EGF receptor was internalized at a low, but significant rate. EGF did not induce down-regulation of this mutant receptor. We also examined the kinetics of both internalization and down-regulation of full length kinase-active and kinase-inactive EGF receptors under the approach, an EGF receptor mutant that lacked a cytoplasmic domain was constructed because information that specifies high efficiency internalization for other receptors is located in this region (Davis et al., 1987; Jing et al., 1990; Verrey et al., 1990). The internalization rate of this "intracellular-sequence-minus" (IS-) receptor should reflect random receptor entrapment by either smooth or coated pits. In the second approach, a monoclonal antibody (mAb 528) was used as a marker to follow empty receptors. This antibody does not activate the receptor and cannot bind to occupied receptors (Gill et al., 1984). This allowed use of a double-label protocol to simultaneously observe both occupied and unoccupied receptors.

We prepared the IS EGF receptor truncation mutant (c'647) which retains only a two-amino acid C' terminus past the transmembrane domain and evaluated the ability of this receptor to both down-regulate and to internalize EGF. As shown in Fig. 1, the IS EGF receptor was internalized at a low, but significant rate. EGF did not induce down-regulation of this mutant receptor. We also examined the kinetics of both internalization and down-regulation of full length kinase-active and kinase-inactive EGF receptors under the

![Fig. 1. Internalization and down-regulation of EGF receptors.](image-url)
same conditions used to evaluate the IS− construction. Kinase activity was required for both efficient internalization and down-regulation (Fig. 1). The average internalization rate of the kinase-active receptor (0.32 min⁻¹) was 8-fold higher than the M²¹⁺ kinase-inactive receptor. Significantly, the internalization rate of EGF bound to full length kinase-inactive receptors was indistinguishable from that observed with the IS− receptor.

The specific internalization rates of anti-EGF receptor antibody bound to wild-type, kinase-inactive, and IS− receptors were all similar (Fig. 1). These rates (0.03-0.05 min⁻¹) were also similar to those displayed by EGF bound to both kinase-inactive and IS− EGF receptors. Concentrations of antibody ranging from 0.1 to 12 nM did not affect internalization rates in cells expressing wild-type EGF receptors (0.03 min⁻¹). In addition, these concentrations of antibody did not induce receptor down-regulation (Fig. 1), in agreement with previous studies using this antibody (Kawamoto et al., 1983).

A statistical summary of receptor internalization rates at low concentrations of EGF and anti-EGF receptor antibody is presented in Fig. 2. These data were collected over a period of 2 years and show the total range of observed values. There was no significant difference (p > 0.05) between the internalization rate of EGF bound to either the full length kinase-inactive or IS− receptors. In addition, there was no difference between the rate of antibody internalization displayed by kinase-active, kinase-inactive, or IS− EGF receptors. The only significant difference was between the internalization rate displayed by kinase-active receptors binding EGF and all other receptors (p < 0.01).

**Induced Internalization of the EGF Receptor Is Saturable—**

To avoid saturation of the specific, coated pit pathway (Wiley, 1988), the internalization data shown in Fig. 2 were collected using 0.2 nM EGF. At this concentration, the internalization rate of wild type EGF receptors was 8-10-fold higher than that of kinase-inactive receptors. Increasing concentrations of EGF resulted in a progressive decrease in the specific internalization rate of the wild type receptor (Fig. 3A). These data are presented as a Satin plot, where the slope is proportional to the affinity of receptors for the endocytic apparatus.

**Specific internalization rate (min⁻¹)**

![Satin plot](image)

**Fig. 2. Statistical summary of the internalization rates of both occupied and empty EGF receptors.** All specific internalization rates of the different receptors were determined using either 0.2 nM EGF or 1 nM 528 monoclonal IgG for the indicated cell types. The data are presented as a box plot in which the center is the median value, while the box encloses the middle 50% of the values, the shaded region is the standard deviation, and the whiskers enclose all the data values. Values in parentheses are the total number of internalization plots from separate experiments included in the analysis.

(Lund et al., 1990b). The Satin plot of the kinase-active receptor was biphasic, indicating entry through both a high affinity and constitutive endocytic pathway (Heisermann et al., 1990; Lund et al., 1990b). The affinity of the high affinity component was 2.5 × 10⁻⁷ M⁻¹ per receptor. Satin plots of the IS− and kinase-inactive receptors were virtually identical and displayed a slope of zero (Fig. 3A), as would be expected of receptors unable to specifically bind to coated pit components. The slope of the Satin plot of wild-type receptors using ²⁵I-labeled 528 IgG as a ligand was also zero (Fig. 3A), indicating that binding of the antibody to unoccupied EGF receptors does not affect their interaction with the endocytic apparatus.

The data in Fig. 3A indicate that the protein tyrosine kinase activity of the EGF receptor is necessary for specific receptor binding to endocytic structures. However, we previously described a kinase-inactive truncation mutant of the EGF receptor (M²¹⁺c'1022) that is able to undergo ligand-induced internalization and down-regulation (Chen et al., 1989; Lund et al., 1990a). To determine whether the M²¹⁺c'1022 mutant was able to undergo ligand-induced binding to the endocytic apparatus, Satin plot analysis was performed using both this mutant and its kinase-active parent receptor (c'1022). As shown in Fig. 3B, the M²¹⁺c'1022 receptor displayed a lower, but significant slope (p < 0.01). In multiple experiments, endocytic affinities were consistently observed to fall between 4.3 and 7.7 × 10⁻⁷ M⁻¹ per receptor, indicating that the M²¹⁺c'1022 mutant EGF receptor has a low affinity for the endocytic apparatus relative to wild type receptors. However, this low affinity was significantly greater than that displayed by IS− or holo kinase-inactive receptors (Fig. 3A). As expected, the kinase-active c'1022 receptor displayed a higher affinity for endocytic structures than the kinase-inactive c'1022 mutant (Fig. 3B).

Satin plots will yield a negative slope for a given receptor type when occupancy increases the internalization rate of the receptor (Lund et al., 1990b). All EGF receptors that display endocytic saturation should thus internalize EGF faster than simultaneously bound 528 antibodies. As shown in Table I, this was observed. Antibody bound to kinase-active or kinase-inactive c'1022 EGF receptors was internalized slightly faster than antibody bound to full length receptors. However, internalization of EGF bound to either c'1022 receptor was significantly faster than antibody internalization (p < 0.01). Al-
Internalization and Down-regulation of the EGF Receptor

TABLE I

Saturation of specific endocytosis is associated with induced internalization

Cells were incubated for 5 min at 37 °C with a combination of ³¹I-EGF and ¹²⁵I-labeled anti-EGF receptor monoclonal antibody 528. The labeled EGF was at the indicated concentration, while the 528 monoclonal antibody was at a concentration of 1.3 nm. Samples were taken at 1-min intervals. The specific internalization rates (k,) of the indicated ligands were determined from internalization plots.

| Receptor type | EGF concentration | ³¹I-EGF | ¹²⁵I-528 IgG |
|---------------|-------------------|---------|-------------|
|               | nM                | min⁻¹    |             |
| WT            | 0                 | 0.05     |             |
|               | 0.8               | 0.26     | 0.06        |
|               | 17                | 0.09     | 0.05        |
| M²⁻¹         | 0                 | 0.04     |             |
|               | 0.8               | 0.03     | 0.04        |
|               | 17                | 0.02     | 0.04        |
| c‘1022        | 0.8               | 0.27     | 0.08        |
|               | 17                | 0.14     | 0.07        |
| M²⁻¹c‘1022    | 0.8               | 0.13     | 0.07        |
|               | 17                | 0.10     | 0.07        |

though high concentrations of EGF could reduce the specific internalization rate of receptors occupied with EGF, there was no effect on the internalization rate of receptors complexed with 528 antibody (Table I). These data are consistent with previous observations that internalization of EGF receptors is a noncooperative process (Wiley and Cunningham, 1982; Waters et al., 1990) and that occupied receptors compete with each other for internalization (Lund et al., 1990b; Wiley, 1988).

Recycling and Degradation of EGF Are Independent of Receptor Tyrosine Kinase Activity—The internalization plot method for determining receptor internalization rates assumes that there is no loss of ligand from within the cell during the time of measurement (Opresko and Wiley, 1987), an assumption that has been validated in other cell types (Dunn and Hubbard, 1984; Wiley and Cunningham, 1982). To ensure that differences between the behavior of kinase-active and -inactive receptors were not due to differences in their rate of recycling or ligand degradation, these parameters were directly measured.

As demonstrated under “Appendix,” the difference between the “true” specific internalization rate (k,) and the “observed” value provided by internalization plots [(k,)sub] is a function of k, and kobs, where k, is the rate constant of recycling and kobs is the rate constant of ligand degradation:

$$\frac{(k_{\text{true}})}{(k_{\text{obs}})} = \frac{(k_{\text{true}} + k_{\text{obs}})}{1 - e^{-k_{\text{true}}t_{\text{rec}}}}$$

(1)

If these parameters are sufficiently small, then measurements of k, will be accurate to within a known degree. However, by measuring ligand recycling and degradation rates, one can correct the “observed” internalization rate.

To determine an upper limit for the recycling rate in transfected B82 cells, we measured the rate and extent of fluid phase diacytosis. Diacytosis, the return of nonspecifically incorporated solute back to the medium, provides an upper limit to the rate at which cells are capable of recycling (Besterman et al., 1981; McKinley and Wiley, 1988). Cells were incubated for 5 and 10 min with [³¹I]PVP and then chased in unlabeled medium. The amount of material remaining with the cells was then measured. As shown in Fig. 4, the cells rapidly retook the [³¹I]PVP back to the medium with an apparent trec of 3.6 min. The rate of diacytosis did not depend on incubation time, but the fraction of fluid phase marker that was retained by the cells increased with the incubation period. These results are consistent with previous studies on fluid phase diacytosis and demonstrate that B82 cells are capable of rapid membrane recycling on the time scale of the experiments. They also set an upper value of k, of approximately 0.19 min⁻¹.

To determine whether receptor-associated ligands were rapidly recycled, we examined the rate at which previously internalized Tf and EGF were returned to the medium. Since Tf rapidly recycles while attached to its receptor, its use as an internal control permitted direct comparison between cells expressing kinase-active and kinase-inactive EGF receptors. EGF was labeled with [³¹I] and Tf was labeled with [¹²⁵I]. Cells expressing either kinase-active or kinase-inactive receptors were exposed to a mixture of both ligands for 2 min followed by a chase in medium containing a high concentration of unlabeled ligands to prevent radiolabeled ligand rebinding. As shown in Fig. 5B, the recycling and discharge of Tf was identical for cells expressing either kinase-active or kinase-inactive EGF receptors. The trec of ligand return in this experiment was approximately 5 min, which was slower than the rate of fluid phase diacytosis. In multiple experiments, the measured time scale of Tf recycling was between 5 and 12 min in B82 cells, which is typical for this receptor (Jing et al., 1990; McGraw and Maxfield, 1990).

EGF was also rapidly lost from the cells, but at a slower rate than Tf. The loss of EGF could be detected within 6 min of the initial ligand exposure, while loss of EGF was detected only after 8 min. In the experiment shown in Fig. 5, EGF internalized by cells expressing kinase-active receptors was lost with a trec of 12 min, while cells expressing kinase-inactive receptors lost EGF with a trec of 25 min. The average of five separate experiments showed a trec of EGF loss of 14 ± 2 and 16 ± 7 min for full length kinase-active and -inactive receptors, respectively. These differences were not statistically significant (p > 0.05). The average recycling rate of EGF internalized by either kinase-active or kinase-inactive c‘1022 truncation mutations was 10 min (data not shown). Thus, although structural features of the EGF receptor could play a role in regulating the rate of recycling, intrinsic kinase activity does not appear to be a primary regulator of this process.
Intracellular Compartmentation of EGF Relative to Transferrin—Although recycling of EGF through the cell was qualitatively similar to that of Tf, it was unclear whether the two ligands were using the same intracellular route. To clarify this issue, cells were incubated for either 5 or 25 min with a combination of 125I-Tf and 125I-EGF. Surface-associated ligand was removed at 0 °C followed by cell homogenization and fractionation. The distribution of EGF and Tf across the endosomal region of the density gradients was then evaluated. As shown in Fig. 6, endosomes were distributed over a relatively symmetrical peak centered at a density between 1.12 and 1.14 g/ml while the plasma membrane was found in the more dense region of the gradient (1.15-1.16 g/ml). The lysosomal marker hexosaminidase was well separated from the endosomes and distributed as a bimodal peak at 1.16 and 1.21 g/ml. Internalized EGF cosedimented with the endosomal Tf after either a 5-min or 25-min incubation, indicating that the same recycling pathway was utilized by both ligands. There was no difference in the compartmentation of EGF in cells expressing either kinase-active or kinase-inactive EGF receptors (Fig. 6), indicating that postendocytic compartmentation is independent of intrinsic receptor tyrosine kinase activity. Although the average density of EGF-containing endosomes shifted to a lower density after 25 min, the same was observed for internalized transferrin (Fig. 6).

To determine whether both Tf and EGF were in the same endocytic vesicles, we utilized the horseradish peroxidase DAB density shift technique (Courtoy et al., 1984; Ajioka and Kaplan, 1987). This method is based on peroxidase-H2O2-catalyzed oxidation of DAB within vesicles. The dense polymer of DAB which forms within the vesicle lumen increases the buoyant density of the vesicle. Thus, peroxidase-containing vesicles can be separated from other vesicles by density gradient centrifugation. Conjugates of HRP and both EGF and Tf were used to place peroxidase activity within endosomes containing the respective receptors. As shown in Fig. 7A, incubation of cells simultaneously with both HRP·Tf and 125I-Tf resulted in a density shift of endosomal 125I-Tf after DAB·H2O2 treatment. This density shift required the presence of HRP·Tf (Fig. 7B). When separate plates of cells were incubated with either HRP·Tf or 125I-Tf and then mixed prior to homogenization, no density shift occurred (Fig. 7B). In a similar manner, endosomes containing both HRP·EGF and 125I-EGF could be shifted to the dense region of the gradient by DAB·H2O2 treatment (data not shown). These data confirm that density shifting occurs only when ligand and HRP are located in the same vesicle (Ajioka and Kaplan, 1987). To demonstrate the colocalization of Tf and EGF within endosomes, cells expressing either kinase-active or kinase-inactive EGF receptors were incubated simultaneously with 125I-EGF and HRP·Tf for 60 min at 0 °C followed by a chase for 15 min at 37 °C. As shown in Fig. 7, C and D, the HRP·Tf was able to shift all endosomally localized 125I-EGF to the bottom of the gradient. Significantly, there was no difference in the ability of HRP·Tf to shift EGF internalized by either the kinase-active or kinase-inactive EGF receptor. These data indicate that the EGF-receptor complex traverses the same recycling endosomal pathway in B82 cells as does the Tf receptor. In addition, the protein tyrosine kinase activity of the EGF receptor does not appear to qualitatively dictate its postendocytic compartmentation.

Relative Extent of EGF Degradation versus Recycling—Loss of previously internalized EGF by the cell could be due to either ligand recycling, rapid degradation, or both. To deter-
The majority of EGF internalized by transfected B82 cells primarily by recycling and not by lysosomal degradation. However, monensin will inhibit intracellular degradation of both EGF (King, 1984; Wiley et al., 1985). As shown in Fig. 8A, there was no measurable EGF degradation by cells expressing kinase-active receptors until 30 min after initial ligand exposure. Cells expressing kinase-inactive receptors displayed significant EGF degradation only after 60 min. The amount of intracellular ligand reached a pseudo steady state prior to significant degradation in either cell type (Fig. 8, B and C), indicating that at steady state, loss of internalized EGF from these cells occurs primarily by recycling and not by lysosomal degradation.

If the amount of internalized EGF in B82 cells is primarily regulated by recycling, then total inhibition of degradation should have relatively little effect on the net intracellular accumulation of ligand. This was tested by treating cells with monensin. It has been previously demonstrated that monensin will inhibit intracellular degradation of both EGF (King, 1984; Wiley et al., 1985) and its receptor (Stoscheck and Carpenter, 1984). Although monensin will also block the recycling of receptors in which ligand dissociation is a requirement for sorting (i.e. the LDL receptor (Basu et al., 1981)), it has little effect on the recycling of membrane lipid (Koval and Pagano, 1989) and a relatively minor effect on other receptor types (i.e. insulin (Huecksteadt et al., 1986) or Tf receptors (Stein and Sussman, 1986)). Fig. 8A shows that 15 μM monensin completely blocks EGF degradation in transfected B82 cells. However, monensin had little effect on either receptor down-regulation or internalization in cells expressing either kinase-active or kinase-inactive EGF receptors (Table II). In addition, we found that monensin had no significant effect on the recycling of internalized EGF, with a t₀ of 16 min after monensin treatment. As expected, blocking EGF degradation did not cause an appreciable net accumulation of intracellular ligand (Fig. 8, B and C). These data confirm that the majority of EGF internalized by transfected B82 cells.
the presence of monensin and either 1.7 nM unlabeled EGF (for the effect of \(^{125}\)I-EGF. Percentage of surface receptors remaining after the 2-h treatment was determined by equilibrium binding of \(^{125}\)I-EGF at 0 °C using untreated cells as the 100% base line.

| Receptor type | Monensin \((\mu M)\) | \(k_r\) \(\text{min}^{-1}\) | Surface receptors remaining |
|---------------|-----------------|-----------------|-----------------|
| WT (Kin\(^{+}\)) | 0 | 0.20 | 39 |
| 15 | 0.17 | 50 |
| M\(^{21}\) (Kin\(^{-}\)) | 0 | 0.04 | 93 |
| 15 | 0.03 | 100 |

follows a recycling rather than a degradative pathway.

To estimate the relative extent of recycling versus degradation of EGF in B82 cells, we simultaneously measured the rates of EGF degradation and internalization as well as surface-associated ligand at steady state (time, >90 min). Degradation was determined by native gel electrophoresis and internalization was determined after the cells reached steady state with unlabeled EGF. For these experiments, cells expressing wild type receptors were incubated with 0.5 nM EGF. At steady state the cells had 13,000 molecules bound to the surface. Their specific internalization rate was 0.27 min\(^{-1}\) and 690 molecules were degraded per minute, as measured by iodotyrosine release into the medium. This translates to a net internalization rate of 3500 molecules per min at steady state during which time less than 700 molecules were degraded. Very similar results were obtained for cells expressing kinase-inactive receptors. Thus for each five molecules of EGF internalized at steady state, one is degraded and four are recycled.

From the above data, the influence of ligand recycling and degradation on the measured specific internalization rate \((k_r)\) can be determined. Although no ligand recycling or degradation could be detected during the initial 5 min after ligand addition, a maximum possible value for both \(k_r\) and \(k_h\) can be set by assuming no delays in recycling and degradation. This yields upper bounds of \(k_r = 0.06 \text{ min}^{-1}\); \(k_h = 0.01 \text{ min}^{-1}\) for the kinase-active receptor and \(k_r = 0.03 \text{ min}^{-1}\); \(k_h = 0.01 \text{ min}^{-1}\) for kinase-inactive receptors. Because internalization was measured over a 5-min period for all cells, these values inserted in Equation 1 yield a \((k_r)_{\text{max}}/(k_h)_{\text{max}}\) Ratio of 1.15 for the kinase-active EGF receptor and 1.09 for the kinase-inactive EGF receptor. Conservatively, the specific internalization rate of the kinase-inactive EGF receptor is thus underestimated by 9% and that of the kinase-active EGF receptor is underestimated by 15%.

**Induced Internalization Is Sufficient to Accelerate Receptor Degradation**—It has been postulated that tyrosine kinase activity of the EGF receptor leads to down-regulation due to phosphorylation of a protein in multivesicular bodies required for lysosomal targeting (Felder et al., 1990). If this is the case, then ligand-induced loss of receptor mass should be dependent on receptor kinase activity, but independent of induced internalization. To critically test this hypothesis, we examined the effect of occupancy on degradation rates of kinase-active EGF receptors truncated to residue 973 (c′973) and kinase-inactive M\(^{21}\)c′1022 receptors. The c′973 receptors retain full tyrosine kinase activity and signal biological responses, but lack a specific domain required for ligand-induced internalization (Chen et al., 1989). Although M\(^{21}\)c′1022 receptors lack kinase activity, they do retain a degree of occupancy-induced internalization (Table I). As shown in Fig. 9A, EGF reduced the cellular mass of M\(^{21}\)c′1022 receptors to a modest extent, concordant with the relative extent of ligand-induced internalization of this receptor (Fig. 3B). This reduction was due to an accelerated degradation rate of the M\(^{21}\)c′1022 receptor as was the case with the holo, kinase-active receptor (Fig. 9B). In contrast, the cellular mass of c′973 receptors was not affected by EGF addition despite their high levels of intrinsic tyrosine kinase activity (Fig. 9A). These data demonstrate that kinase activity is not required for occupancy-induced receptor degradation and thus phosphorylation of a protein in multivesicular bodies cannot be a requirement for lysosomal targeting. However, kinase activity leads to an increased loss in receptor mass in parallel with effects on internalization rates. This indicates that receptor degradation is proportional to the pool of intracellular receptors which in turn is regulated primarily by receptor internalization.

**DISCUSSION**

A basic question regarding the diverse classes of membrane receptors that possess intrinsic tyrosine kinase activity is the molecular mechanisms by which they are down-regulated. While it has been firmly established that the intrinsic tyrosine kinase activity of the EGF receptor is required for biological activity (Chen et al., 1987; Honegger et al., 1987b), it has been less clear whether this enzymatic function is required for ligand-induced internalization and postendocytic targeting (Chen et al., 1989; Felder et al., 1990; Glenney et al., 1988;

![Fig. 9. Ligand-induced degradation of EGF receptors. A, Western blot analysis of total cell EGF receptor mass. Cells expressing the indicated receptors were treated without or with 50 nM EGF for 24 h prior to analysis. Receptor mass was determined by densitometry and is expressed as the ratio of EGF-treated to control using the average of duplicate experiments. Arrows indicate the position of immunostained mutant EGF receptors. B, pulse-chase analysis of the effect of EGF on degradation of holo kinase-active (○, O) and M\(^{21}\)c′1022 kinase-inactive (△, △) EGF receptors. Triplicate wells from EGF-treated (○, △) and control (●, ▲) cultures were measured. Values for \(t_0\) of receptor degradation were: holo receptor 48 and 15 h and M\(^{21}\)c′1022 receptor 52 and 17 h without and with 100 nM EGF, respectively.](image)
Honegger et al., 1987a, 1990). The current study provides several independent lines of evidence that intrinsic tyrosine kinase activity is required for rapid endocytosis of the holo EGF receptor, but does not regulate recycling or lysosomal targeting.

There are three possible fates of all cell surface receptors with respect to coated pit internalization. Receptors can be specifically included in coated pits, they can be excluded from those structures, or they can be neither included nor excluded. In the last case, the receptors should be internalized at a rate that reflects random capture by invaginating endocytic structures. This rate can be estimated by considering the fastest reported internalization rates of receptors. If a receptor is 100% captured by coated pits, then its specific internalization rate will equal that of the coated pit itself (Goldstein et al., 1984). Carbohydrate-binding receptors have among the fastest internalization rates, usually ranging between 1.2 and 1.3 min⁻¹, with the fastest reported rate being 4.1 min⁻¹ (Ward and Kaplan, 1990; Magnusson and Berg, 1989). Because coated pits comprise about 2% of the cell surface, random entrapment by coated pits should yield internalization rates of between 0.03 and 0.08 min⁻¹. If random capture by smooth pits is included, the estimate is even higher. Recently, specific internalization rates of Tf receptors lacking either a cytoplasmic tail (Jing et al., 1990) or a specific internalization sequence (McGraw and Maxfield, 1990) have been estimated, yielding values between 0.04 and 0.06 min⁻¹. Similarly, replacing the cytoplasmic tail of the avian asialoglycoprotein receptor with unrelated sequences from Xenopus globin produced receptors with internalization rates between 0.02 and 0.06 min⁻¹ (Verrey et al., 1990). Finally, all “internalization-defective” LDL receptors are internalized at a rate between 0.02 and 0.03 min⁻¹ (Davis et al., 1987). Because these mutant LDL receptors are highly mobile in the plasma membrane (Barak and Webb, 1982; Goldstein et al., 1984), but are unable to cluster in coated pits (Beisiegel et al., 1981), their internalization rate should accurately reflect random entrapment by coated pits or other endocytic structures. All these data suggest that the specific internalization rate of randomly entrapped receptors will fall somewhere between 0.02 and 0.06 min⁻¹. This is precisely the range of values we observed for kinase-inactive receptors (~0.03 min⁻¹), unoccupied receptors, and receptors lacking a cytoplasmic domain.

The specific internalization rate of the ligand-bound, kinase-active receptors at low occupancies averaged 0.32 min⁻¹, indicating that 32% of the occupied receptors were internalized per minute. This value declined to 0.06 min⁻¹ as the endocytic system became saturated at high levels of receptor occupancy. In contrast, the internalization rate for ligand-occupied, kinase-inactive receptors was 0.03 min⁻¹ at all levels of occupancy. The low internalization rate of kinase-inactive receptors was also shared by EGF receptors lacking a cytoplasmic domain (IS-) and by unoccupied wild type receptors as measured by antibody internalization. Therefore, the large differences in endocytic behavior between kinase-active and kinase-inactive EGF receptors appear to be explained by the ability of kinase-active EGF receptors to bind with high affinity to coated pits or other components of the endocytic apparatus. EGF receptor truncation mutants c1022 which lack intrinsic tyrosine kinase activity display occupancy-facilitated internalization to a lesser extent than the holo, kinase-active receptor, and they display a relatively low affinity for the endocytic system. However, kinase-active c1022 EGF receptor mutants have a significantly greater affinity for the endocytic apparatus and undergo internalization and down-regulation with a corresponding greater efficiency. All receptors that displayed a ligand-induced increase in endocytic rates appear able to saturate the endocytic system and to down-regulate (Chen et al., 1989).

Together, these data lead to the following model. In the absence of tyrosine kinase activity (empty receptors or occupied receptors modified by site-directed mutagenesis), EGF receptors are internalized by random entrapment in endocytic structures. Ligand binding leads to a conformation change that allows interaction with coated pit components. Because ligand binding activates intrinsic protein tyrosine kinase activity, phosphorylation of some component by the EGF receptor could stabilize this interaction, leading to the observed high affinity binding to the endocytic apparatus. Receptors modified by site-directed mutagenesis could bind either more tightly or less tightly to coated pits, depending on the structural consequence of the modification. However, “ligand-induced” internalization and subsequent receptor down-regulation can only occur when receptor affinity for endocytic structures is significantly increased by occupancy. Since C terminal sequences are required for ligand-induced internalization of EGF receptors (Chen et al., 1989), kinase-active receptors which lack these sequences neither undergo ligand-induced down-regulation nor loss of receptor mass.

Other investigators have postulated that tyrosine kinase activity is required for EGF receptor down-regulation due to a kinase-mediated block in receptor recycling rather than at the level of ligand-induced internalization (Honegger et al., 1987a, 1990; Felder et al., 1990). Despite extensive investigations, we could find no evidence to support this claim. Careful, direct measurement of the rate and extent of EGF recycling in cells expressing either kinase-active or kinase-inactive receptors indicates that EGF recycling is very similar for both receptors. Further, cell fractionation and density shift colocalization experiments demonstrate no qualitative difference between the postendocytic compartmentation of kinase-active or kinase-inactive receptors. Honegger et al. (1987a, 1990) and Felder et al. (1990) also failed to observe kinase-dependent EGF receptor internalization. Analysis of their data reveals that the internalization rate for the kinase-inactive receptor was between 0.02 and 0.06 min⁻¹, well within the range reported here. However, the apparent internalization rate of the wild-type receptor in their experiments, was only about 0.10 min⁻¹ (Honegger et al., 1987a), somewhat below the range of 0.14–0.5 min⁻¹ reported for the EGF receptor in a wide variety of cell types (Gex-Fabry and DeLisi, 1988; Gill et al., 1988; Lloyd and Ascoli, 1983; Waters et al., 1990; Wiley and Cunningham, 1982). An inspection of their technique for measuring internalization rates (Honegger et al., 1987a, 1990) shows that conditions that favor high numbers of occupied receptors were used. This would bias estimates of specific internalization rates of wild-type receptors to lower values (Benveniste et al., 1988; Wiley, 1988; Chen et al., 1989; Lund et al., 1990b). In addition, their technique measures net internalization rates (total per cell) rather than specific internalization rates (rate per receptor). Net internalization rates of cells undergoing down-regulation will always fall as a function of time relative to cells maintaining constant surface receptor number, narrowing the initial difference between kinase-active and kinase-inactive receptors.

Interestingly, the postendocytic trafficking pattern of EGF and its receptor appears to be highly cell-type dependent. Although the EGF receptor recycles efficiently in transfected B82 cells and other types (Dunn and Hubbard, 1984; Kore and Magun, 1985; Murthy et al., 1986; Sorkin et al., 1989), there appears to be little if any EGF receptor recycling in human fibroblasts (Stosscheck and Carpenter, 1984). Recy-
The Effect of Ligand Recycling and Degradation on Internalization Analysis—We derive a conservative estimate of the effects of receptor/ligand recycling and degradation on $k_r$ values obtained from short-time internalization experiments. The starting point is a kinetic species balance equation for the number of intracellular ligand/receptor complexes at time $t$, $[LR](t)$ as follows,

$$\frac{d[LR]}{dt} = k_i[LR] - (k_h + k_d)[LR].$$

(A-1)

where $[LR]$ is the number of complexes on the surface at time $t$, $k_i$ is the complex endocytic rate constant, $k_h$ is the receptor recycling rate constant, and $k_d$ is the intracellular degradation rate constant.

The solution to this differential equation can be obtained through a variation-of-parameters approach (see, for example, Ritger and Rose, 1968),

$$[LR](t) = \int_0^t k_i[LR](-\tau) e^{-\int_0^\tau (k_h + k_d) \, d\tau} \, d\tau$$

(A-2)

where $\tau$ is a dummy variable for integration. This solution has a clear physical interpretation. For any number of complexes on the surface at a given time $\tau$, the corresponding number of intracellular complexes at a later time $t$ will be equal to the product of the internalization rate for those original cell surface complexes and the “decay” of the intracellular complexes due to recycling and/or degradation during the intervening time period ($t - \tau$).

To calculate the precise effect of recycling and/or degradation on an estimate for $k_r$, we would need to know the entire time-history of the surface complex number $[LR](t)$. This requires writing kinetic species balance equations for that quantity along with all other quantities involved, such as surface free receptor number, intracellular free receptor number, and extracellular and intracellular free ligand concentrations. This will be presented elsewhere, but more insight can be gained from an approximate estimate of the effect of interest. We note that the predictions from this estimate are consistent with, and in fact, conservative when compared with the more comprehensive model computation results.

Recycling and/or degradation will have their greatest effect on the number of internalized complexes, $[LR](t)$, when that number is as large as possible. This is because recycling and degradation processes occur at rates proportional to that number. In our experiments, the number of surface complexes, $[LR](t)$, builds up from an initial value of 0 at $t = 0$ to a quasi-steady state value once the receptor/ligand binding and dissociation processes reach a dynamic balance (Wiley and Cunningham, 1981). This value is called $[LR]_{\text{ss}}$. $[LR](t)$ will correspondingly increase from an initial value of 0 as $[LR]_{\text{ss}}$ increases. The value of $[LR](t)$, and thus the effects of recycling/degradation, will be conservatively overestimated if we assume that $[LR](t) = [LR]_{\text{ss}}$ for all $t$. This is the assumption made to derive the analytical estimate.

With this assumption on $[LR]$, the solution expressed in Equation A-2 simplifies to the following.

$$[LR](t) = \frac{k_i[LR]_{\text{ss}}}{k_h + k_d} e^{-k_h t}$$

(A-3)

The Wiley-Cunningham method for producing an “observed” value of $k_r$ neglects effects of recycling and degradation during

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APPENDIX

The Effect of Ligand Recycling and Degradation on Internalization Analysis—We derive a conservative estimate of the
short-time internalization experiments (Wiley and Cunningham, 1982), and uses the expression as follows.

\[
(k)_{\text{raw}} = \frac{[LR]_{\infty}}{[LR]_{\text{af}}} \left( k + k_r \right) t \left( 1 - e^{-k_r t} \right) \quad (A-5)
\]

At the same time the "true" value for \( k \) in this case should be as follows.

\[
(k)_{\text{raw}} = \frac{[LR]_{\infty}}{[LR]_{\text{af}}} (k + k_r) t \left( 1 - e^{-k_r t} \right) \quad (A-6)
\]

Hence, the ratio we desire is the following.

\[
\frac{(k)_{\text{raw}}}{(k)_{\text{af}}} = \frac{(k + k_r) t}{1 - e^{-k_r t}} \quad (A-7)
\]

Note that for experiments of duration \( t \ll (k_r + k_r)^{-1} \), this ratio reduces to 1. Thus there is an explicit, quantitative definition for the time period during which recycling and degradation can be safely neglected. This result will allow a posteriori confirmation of the validity of this neglect once \( k_r \) and \( k_r \) are determined. For experiments of very long duration, so that \( t \gg (k_r + k_r)^{-1} \), this ratio approaches \((k_r + k_r)(t)\) and thus becomes linearly proportional to time. This results from the fact that, for these long time periods, the system as defined by Equation 1 achieves a steady state at which the ratio \([LR]_{\infty}/[LR]_{\text{af}}\) is equal to the quantity \(k/k_r+k_r\), while the Wiley-Cunningham analysis of internalization rates does not permit a steady-state to be achieved (since it has neglected recycling and degradation). The applicability of such an approach clearly breaks down at such long time periods.

Methods for determining \( k \) have been previously described by a number of different investigators (Wiley and Cunningham, 1981; Myer et al., 1987; Waters et al., 1990). An estimate of \( k \) can be determined from the chase portion of a pulse-chase curve such as shown in Fig. 5, with \((k)_{\text{af}} = (ln 2)/t_\text{chase}\). Analysis of the relative influence of reendoctyosis of recycled complexes during the chase period allows correction of \((k)_{\text{af}}\) to \((k)_{\text{raw}}\) in similar fashion to the correction of \((k)_{\text{af}}\) to \((k)_{\text{raw}}\) for the internalization experiments. Although a detailed derivation will be presented elsewhere, a good approximation is as follows.

\[
\frac{(k)_{\text{raw}}}{(k)_{\text{af}}} = 1 + \frac{(k)_{\text{af}} + (k)_{\text{inv}}}{k_d} \quad (A-8)
\]

A condition necessary for this expression to be valid is \(4k_d k_r < (k_r + k_r)^{-1}\). This is easily satisfied when the dissociation rate constant is large compared to the recycling rate constant. In the case of both the kinase-active and inactive EGF receptors, \( k_d \sim 10^{-3} \) fold higher than \( k_r \) (data not shown). The relationship quantified in Equation A-8 is intuitively reasonable since the correction will be small when dissociation is fast compared to recycling and reendoctyosis, since a complex reappearing at the cell surface will dissociate before having the chance to be internalized again.

The corrections expressed in Equations A-7 and A-8 both involve \( k \) and \( k_r \) in coupled fashion. Hence, they can be applied iteratively or simultaneously. In either manner, good estimates for the true values can be obtained from the original observed values. The most important point to note is that both corrections will be greater (and upward) for the kinase-active receptor in comparison to the kinase-inactive EGF receptor because both \( k \) and \( k_r \) appear larger in the case of the former. Therefore, the comparative differences between the true values of these parameters for the two receptor types will be at least as great, and likely greater, than those between the observed values.

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