Recruitment of Epidermal Growth Factor Receptors into Coated Pits Requires Their Activated Tyrosine Kinase

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Abstract. EGF-receptor (EGF-R) tyrosine kinase is required for the down-regulation of activated EGF-R. However, controversy exists as to whether ligand-induced activation of the EGF-R tyrosine kinase is required for internalization or for lysosomal targeting. We have addressed this issue using a cell-free assay that selectively measures the recruitment of EGF-R into coated pits. Here we show that EGF bound to wild-type receptors is efficiently sequestered in coated pits. In contrast, sequestration of kinase-deficient receptors occurs inefficiently and at the same basal rate of endocytosis of unoccupied receptors or receptors lacking any cytoplasmic domain. Sequestration of deletion mutants of the EGF-R that lack autophosphorylation sites also requires an active tyrosine kinase. This suggests that a tyrosine kinase substrate(s) other than the EGF-R itself, is required for its efficient ligand-induced recruitment into coated pits. Addition of a soluble EGF-R tyrosine kinase fully and specifically restores the recruitment of kinase-deficient EGF-R into coated pits providing a powerful functional assay for identification of these substrate(s).

THE binding of EGF to its cell surface receptor triggers intracellular signaling pathways through a well-defined sequence of events to regulate cell proliferation and development (Cantley et al., 1991). Upon ligand binding, EGF-receptors (EGF-R)[1] dimerize and the intrinsic tyrosine kinase becomes activated leading to autophosphorylation of the receptor. Specific intracellular substrates involved in the metabolic and mitogenic responses to EGF are recruited to the newly created phosphotyrosine-containing binding sites on the EGF-R via their SH2 domains (Carpenter, 1992). EGF binding also triggers efficient receptor internalization. Internalized receptors are segregated from the default recycling pathway and are routed to lysosomes where they are degraded. The resulting decrease in the number of receptors expressed at the cell surface is termed receptor down-regulation (Haigler et al., 1979). Down-regulation of activated EGF-R is believed to play an essential role in modulating the cell's proliferative response to the growth hormone (Wells et al., 1990).

Mutation of the intrinsic tyrosine kinase activity of the EGF-R abolishes both signaling for biological responses and ligand-induced down-regulation (Chen et al., 1987; Honneger et al., 1987). The question of whether the tyrosine kinase of the EGF-R is directly required for its internalization remains disputed (Sorkin and Waters, 1993). Comparable studies on EGF internalization in transformed cells expressing wild-type or kinase-deficient mutant EGF-R have led to contradictory conclusions. Some studies reported that kinase-deficient receptors are not efficiently internalized (Glenney et al., 1988; Chen et al., 1989; Wiley et al., 1991). Others argue that kinase-deficient receptors are internalized as efficiently as wild-type receptors in response to EGF (Honneger et al., 1987; Felder et al., 1992) and instead suggest that the EGF-R tyrosine kinase activity prevents receptor recycling and is required to target internalized EGF-R to lysosomes for degradation (Felder et al., 1990; Honneger et al., 1990). One contributing factor in this debate stems from the difficulty in directly measuring the relative contributions of the rates of EGF-R endocytosis and recycling to the overall initial rate of accumulation of EGF in intact cells.

To clarify this controversial issue, we studied the internalization of EGF in a novel cell-free assay that specifically and directly measures only the early events of endocytosis induced by EGF. This system uses perforated cells that are prepared by mechanical disruption so as to leave large gaps in their otherwise intact plasma membrane (Smythe et al., 1992). Cytosolic contents can then be removed allowing full access to exogenously added reagents. The recruitment of receptor-bound biotinylated EGF (B-EGF) into deeply invaginated, constricted coated pits is detected as it becomes inaccessible to exogenously added avidin (Lamaze et al., 1993). We have previously shown by both biochemical and morphological criteria, that the sequestration of B-EGF into constricted coated pits requires cytosolic factors that are distinct from those required for the efficient sequestration of the

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1. *Abbreviations used in this paper*: B-EGF, biotinylated-EGF; B-Tfn, biotinylated-Tfn; EGF-R, EGF receptor; Tfn, transferrin; wt, wild type.

constitutively internalized transferrin receptor. In addition, the sequestration of EGF but not transferrin (Tfn) is inhibited by the tyrosine kinase inhibitor, genistein. Using this assay we show that kinase-deficient receptors fail to undergo ligand-induced sequestration into coated pits. Sequestration of truncated EGF-R lacking the regulatory domain and its autophosphorylation sites also requires an active EGF-R tyrosine kinase. These results suggest that a kinase substrate(s), in addition to the EGF-R itself, is required for ligand-induced recruitment of activated EGF-R into coated pits. Finally, efficient recruitment of kinase-deficient EGF-R can be restored in trans by the addition of a soluble constitutively active EGF-R kinase. These results provide the first biochemical evidence that an activated tyrosine kinase is essential for the efficient ligand-induced recruitment of EGF-R into coated pits.

Materials and Methods

Cells and Reagents

Stably transformed B82 mouse L cells expressing either wild-type or mutant human EGF receptors were obtained from G. N. Gill (University of California, San Diego, CA) and have been characterized elsewhere (Chen et al., 1989). B82L cells were grown in Dulbecco's modified Eagle medium containing 10% dialyzed calf serum and 1% non essential amino acids (GIBCO BRL, Gaithersburg, MD) and 5 μM methotrexate. The soluble EGF-R cytoplasmic was obtained from D. Cadena (Gill laboratory, University of California, San Diego, CA) and was purified as previously described (Wedegaertner and Gill, 1989).

Receptor-mediated Endocytosis in Intact Cells

B82L cells were grown to confluence on a 6-cm dish and dissociated for 5 min at 37°C in PBS containing 5 mM EDTA. Cells were harvested, washed in 10 ml PBS, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and pelleted (1100 *g* for 5 min). The cells were then resuspended in 1 ml of PBS, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, 0.2% bovine serum albumin containing 20 ng/ml B-EGF (Boehringer Mannheim Corp., Indianapolis, IN), and 2 μg/ml biotinylated-transferrin (B-Tfn), prepared as previously described (Smythe et al., 1992). 40 μl of this cell suspension (containing $\sim 10^5$ cells) were incubated for the indicated times at 37°C before returning to ice. Sequestration of B-EGF was determined after masking surface-exposed B-ligands with avidin and quantitating inaccessible biotinylated ligands as previously described (Smythe et al., 1992; Lamaze et al., 1993). Briefly, cells were pelleted and resuspended at 4°C in a solution of avidin (50 μg/ml). After quenching excess avidin with biocytin (0.5 mg/ml), cell lysates were prepared and plated on microtitre plates coated with either anti-EGF or anti-Tfn antibodies. Internalized B-ligand was then quantitated using streptavidin-HRP (Boehringer Mannheim Corp.).

Sequestration Assay in Perforated Cells

Perforated B82L cells were prepared as described for A431 cells except that an additional "freeze-thaw" step was introduced as follows. Cells were scraped from confluent 15-cm dishes and depleted of endogenous cytosol by incubation in KSHM buffer (100 mM KOAc, 85 mM sucrose, 20 mM Hepes, 1 mM $MgCl_2$, pH 7.3) for 10 min at 4°C. After pelleting (1100 *g* for 5 min), the cells were gently resuspended in 2× vol of 0.75 M sucrose, 5 mM $MgCl_2$, 20 mM Hepes (pH 7.3), and quickly frozen in liquid N_2. Aliquots of frozen cells could be stored for up to 2 mo at −70°C without appreciable loss of activity. For assays, the cells were thawed quickly at 37°C in 2× vol of 120 mM KOAc, 1 mM $MgCl_2$, 20 mM Hepes (pH 7.3). As soon as the ice was melted, the cells were distributed into microfuge tubes (10^5 cells/assay) at 4°C into KSHM assay buffer containing K562 cytosol (6 mg/ml), an ATP-regenerating system, 0.2% BSA, 20 ng/ml B-EGF and 2 μg/ml B-Tfn as described (Lamaze et al., 1993). After transfer for the indicated times to 37°C, the tubes were returned to ice for determination of B-ligand sequestration as described above. All assays were performed in duplicate the results from which differed by <5%.

Determination of the Basal Rate of Endocytosis

The antagonistic monoclonal anti-EGF-R antibody, mAb528 was obtained from G. Gill and used to follow the basal rate of sequestration of unoccupied EGF-R as described elsewhere (Gill et al., 1984; Wiley et al., 1991). mAb528 was biotinylated using NHS-LC-biotin (Pierce Chem. Co., Rockford, IL) as follows: 2 μl of NHS-LC-biotin (6.5 mM in dimethyl sulfoxide), corresponding to a 10-fold molar excess, was added to 100 μl of 2 mg/ml mAb528 in PBS and incubated for 60 min at room temperature. Unconjugated NHS-LC-biotin was removed by gel filtration on a Sephadex G-25 spin-desalt column equilibrated in PBS containing 0.2% BSA. Biotinylated mAb528 (1 μg/μl) was aliquoted, frozen in liquid nitrogen, and stored at −70°C. Assays were performed as described above. B-EGF (20 ng/ml) and B-mAb528 (5 μg/ml) were added to the same reaction. Control experiments showed that at these subsaturating concentrations, the two ligands do not interfere with each other. After processing for avidin inaccessibility, the cells were lysed in a final volume of ~210 μl. 100-μl aliquots of the cell lysate were plated into microtitre wells containing 0.1 ml blocking buffer and coated with either anti-EGF or anti-IgG antibodies. ELISA plates to detect EGF were prepared as described (Lamaze et al., 1993). ELISA plates to detect B-mAb528 were prepared following the same procedure except that rabbit anti-mouse Ig G (Cappel Labs., Cochranville, PA) was plated onto immuno-module strips at a 1 μg/ml in 50 mM Na_2HCO_3, pH 9.6. The plates were then treated as described (Carter et al., 1993).

Results

EGF Is Inefficiently Accumulated in Intact Cells Expressing Kinase-deficient EGF-R

Mouse B82L fibroblasts expressing either wild-type or mutant human EGF-R were used to determine whether an active tyrosine kinase was required for receptor internalization. The EGF-R mutants employed are diagrammed in Fig. 1 and included a kinase-deficient receptor that contained a point mutation (K^{721}→M) in the ATP-binding site (designated kin$^-$) and a truncated receptor that lacked all but three residues of the cytoplasmic domain (designated Δtail). Internalization of receptor-bound biotin-EGF (B-EGF) conjugates into intact or mechanically perforated cells was followed by measuring its acquired inaccessibility to exogenously added avidin (Smythe et al., 1992; Lamaze et al., 1993). After masking surface-exposed B-EGF with avidin and quenching excess avidin with biocytin, lysates were prepared and plated on microtitre wells coated with anti-EGF antibodies. Internalized B-EGF was then quantitated using streptavidin-HRP. B-EGF was efficiently and rapidly ($t_{1/2}$ ~5 min) accumulated in intact B82L cells expressing wild-type (wt) EGF-R (Fig. 2, *closed circles*). In contrast, the accumulation of B-EGF into B82L cells expressing either kin$^-$ or Δtail EGF-R mutants (Fig. 2, *open symbols*) was slow and considerably less efficient than for wt receptors.

EGF Is Inefficiently Sequestered into Coated Pits in Perforated Cells Expressing Kinase-deficient EGF-R

The observed differences in rates and extents of intracellular accumulation of receptor-bound EGF in intact cells are in good agreement with previous findings by other groups (Wiley et al., 1991; Felder et al., 1992). It has been proposed that these differences could be due to either early events in internalization (Chen et al., 1989) or to changes in the recycling efficiency of internalized receptor–ligand complexes (Felder et al., 1990). To distinguish these two possibilities we compared the efficiency of sequestration of receptor-bound B-EGF in perforated B82L cells expressing mutant

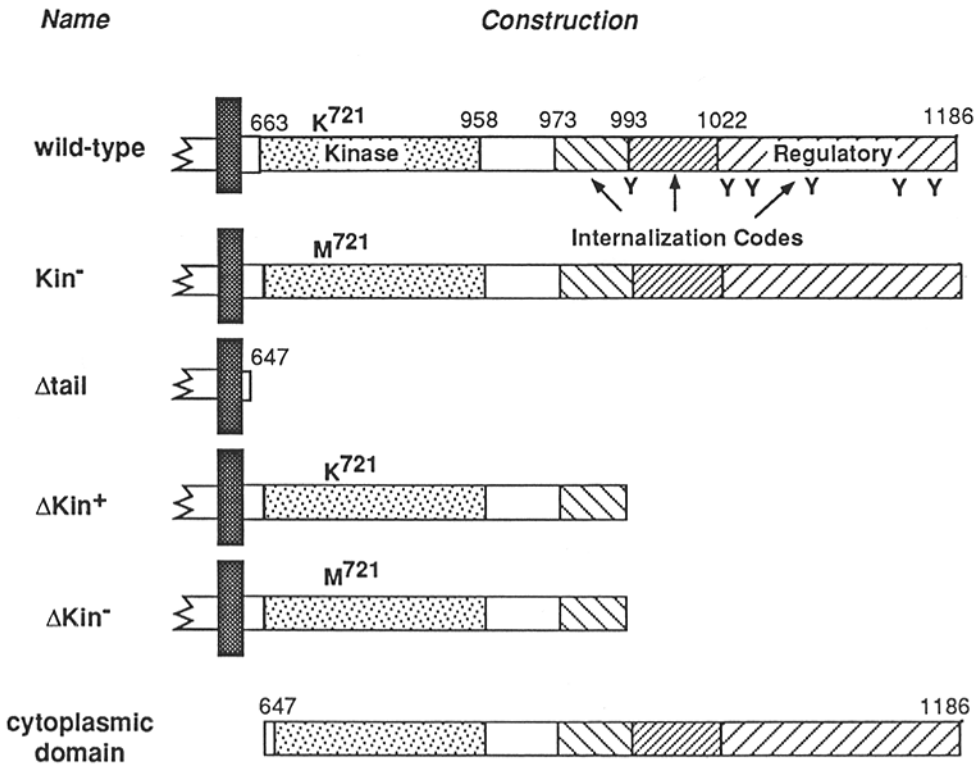

Figure 1. Schematic representation of EGF-R constructs used in this study. Stably transformed B82 mouse L cells (Chen et al., 1989) used in this study expressed either the wild-type or mutant human EGF receptors diagrammed here. Kinase-deficient receptors have a lys^{721} to met point mutation in the ATP-binding site that inactivates the kinase (Chen et al., 1987). Truncated receptors designated Δtail, lack all but three residues of their cytoplasmic tail. Truncated receptors lacking the regulatory domain and containing one of three endocytic codes spliced to an active or defective kinase domain are designated Δkin^+ or Δkin^-, respectively. The EGF-R cytoplasmic domain was expressed as a soluble, active kinase in Sf9 insect cells using the baculovirus expression system (Wedegaertner and Gill, 1989).

and wild-type receptors. Consistent with our previous extensive characterization of this system (Schmid and Smythe, 1991; Smythe et al., 1992; Carter et al., 1993; Lamaze et al., 1993), EGF-receptor recycling does not occur in these perforated cells since there is no detectable increase in total cell-associated EGF-R with time of incubation (data not shown). Furthermore, previous biochemical and morphological studies established that cell surface EGF-R fail to be internalized into sealed coated vesicles and instead accumulate in constricted coated pits (Lamaze et al., 1993). Thus this assay enables us to selectively measure only the early events in EGF-R endocytosis; namely, the recruitment and sequestration of receptor-bound EGF into deeply invaginated, constricted coated pits (Lamaze et al., 1993). In this and all experiments, Tfn sequestration was used to follow the efficiency of constitutive receptor-mediated endocytosis within the same perforated cells and serves as an internal control for the integrity of the endocytic activity of each membrane preparation. As shown in Fig. 3 *A*, Tfn sequestration was equally efficient (40–50% of total cell associated ligand) in perforated B82L cells expressing either wt or mutant EGF-Rs. Similarly, the data in Fig. 3 *B* (*closed circles*) shows that the sequestration of EGF bound to wild-type EGF-R was efficiently reconstituted in perforated B82L cells. The ATP- and cytosol-dependent sequestration of B-EGF corresponds to 60–80% of what was observed in intact cells and was comparable to the efficiency of Tfn sequestration. In contrast, the sequestration of B-EGF was severely impaired in perforated cells expressing either kin^- or Δtail EGF-R mutants (Fig. 3 *B*, *open symbols*). Sequestration of B-EGF in perforated cells expressing mutant receptors was impaired to quantitatively the same extent as was B-EGF accumulation in intact cells. These data suggest that early events in internalization of the EGF-R require its tyrosine kinase activity.

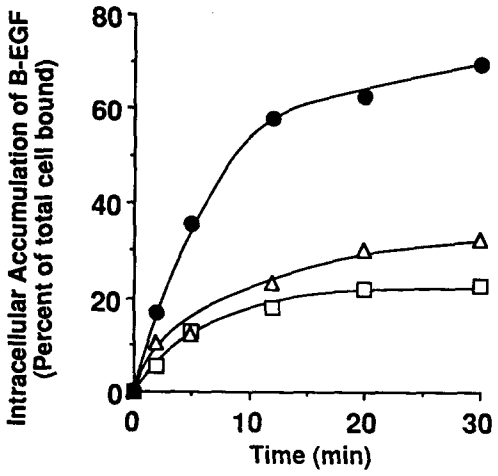

Figure 2. Receptor-mediated endocytosis of wild-type and mutant EGF-R in intact B82L cells. Time course of the intracellular accumulation of B-EGF in intact B82L cells expressing either wt (●), kin^- (□), or Δtail (△) EGF-R. Intact B82L cells were incubated for the indicated times at 37°C in the presence of 20 ng/ml B-EGF before returning to ice. Sequestration of B-EGF was determined after masking surface-exposed B-ligands with avidin and quantitating inaccessible biotinylated ligands as described in Materials and Methods. All assays were performed in duplicate the results from which differed by <5%. The background value of internalization at 4°C (~8% total) has been subtracted from the results shown.

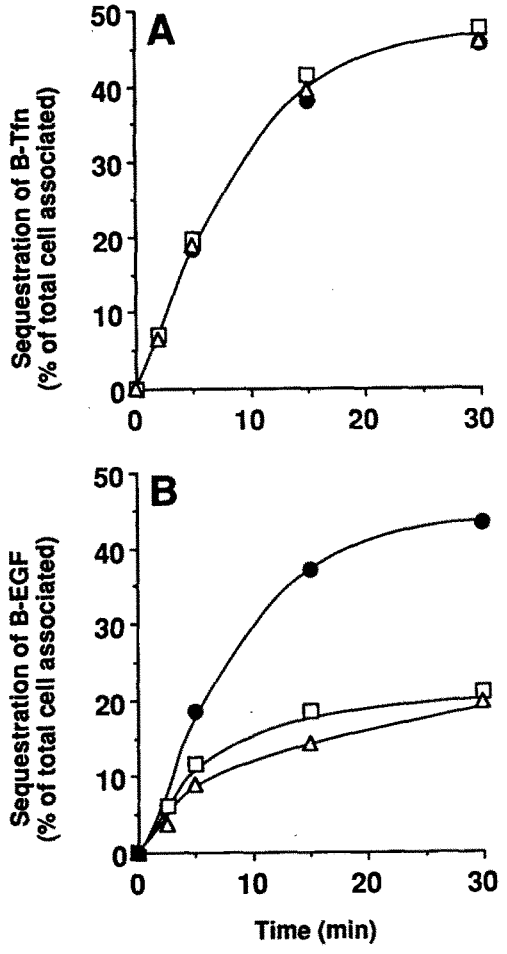

Figure 3. Sequestration of Tfn and EGF in perforated B82L cells expressing wild-type and mutant EGF-R. Time course of the sequestration of B-Tfn (*A*) or B-EGF (*B*) in perforated B82L cells expressing either wt (●), kin⁻ (□), or Δtail (△) EGF-R. Perforated cells were prepared and incubated for the indicated times at 37°C in the presence of K562 cell cytosol, an ATP regenerating system and 20 ng/ml B-EGF before returning to ice and determining the extent of sequestration of B-EGF as described in Materials and Methods. All assays were performed in duplicate the results from which differed by <5%. The data for this and all subsequent experiments using perforated cells shows the cytosol and ATP-dependent sequestration of EGF, expressed as the percent of total cell-associated B-EGF. For this experiment, the A_{490} obtained in wild-type cells after a 2-min incubation to detect streptavidin-HRP was 0.501 and 0.711 for total cell-associated B-EGF and B-Tfn, respectively. It was 0.540 and 0.720 in kin⁻ cells and 0.620 and 0.770 in Δtail cells. Backgrounds (corresponding to 10–15% of total cell-associated ligand) obtained from perforated cells incubated at 37°C for 30 min in the presence of an ATP-depleting system and in absence of cytosol have been subtracted.

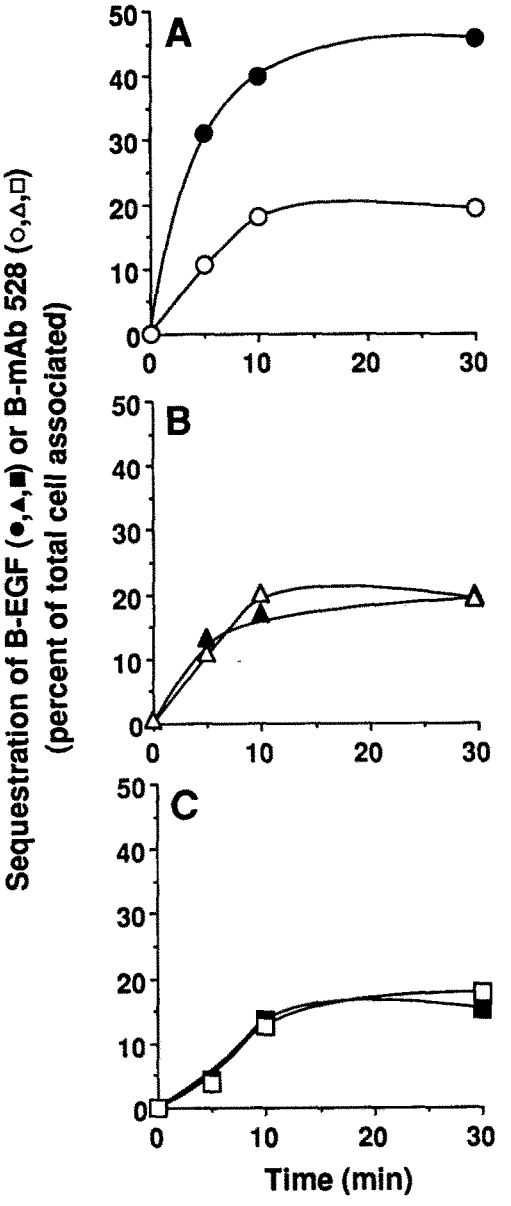

Figure 4. Comparison of the basal and ligand-induced rates of EGF-R sequestration in perforated cells. The antagonistic monoclonal anti-EGF-R antibody, mAb528 was biotinylated and used to follow the basal rate of sequestration of unoccupied EGF-R. The rates of sequestration of B-EGF (*closed symbols*) and B-mAb528 (*open symbols*) in perforated B82L cells expressing either wt (*A*), Δtail (*B*), or kin⁻ (*C*) EGF-R are shown. Assays were performed as described in Fig. 3 except that B-EGF (20 ng/ml) and B-mAb528 (5 μg/ml) were added to the same reaction. Control experiments showed that at these subsaturating concentrations, the two ligands do not interfere with each other. After processing for avidin inaccessibility, the cells lysate was divided equally and plated into microtitre wells containing either anti-EGF or anti-IgG antibodies as described in Materials and Methods.

Mutant Receptors Are Internalized at the Basal Rate

Although ligand binding triggers the rapid internalization of EGF-R, unoccupied EGF-R are nonetheless internalized at a low basal rate (Chen et al., 1989). The data in Figs. 2 and 3 *B* shows that mutant EGF-R are also sequestered at measurable, albeit slow rates. To determine whether mutant EGF-R display any residual ligand-induced internalization, we measured the basal rate of sequestration of unoccupied EGF-R using the competitive antagonistic antibody mAb528 as ligand (Gill et al., 1984; Wiley et al., 1991). Biotinylated mAb528 bound to wt EGF-R was sequestered into perforated cells at a basal rate that was two- to threefold less efficient than ligand-induced sequestration of EGF (Fig. 4 *A*, *open circles*). As expected, the rate of sequestration of EGF

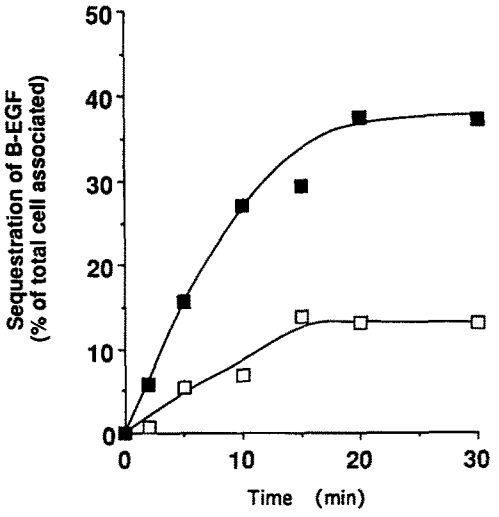

Figure 5. Sequestration of EGF in perforated B82L cells expressing Δkin^+ and Δkin^- EGF-R. Time course of the sequestration of B-EGF in perforated B82L cells expressing either Δkin^+ (■) or Δkin^- (□) EGF-R. Perforated cells were prepared and incubated for the indicated times at 37°C as described in Fig. 3.

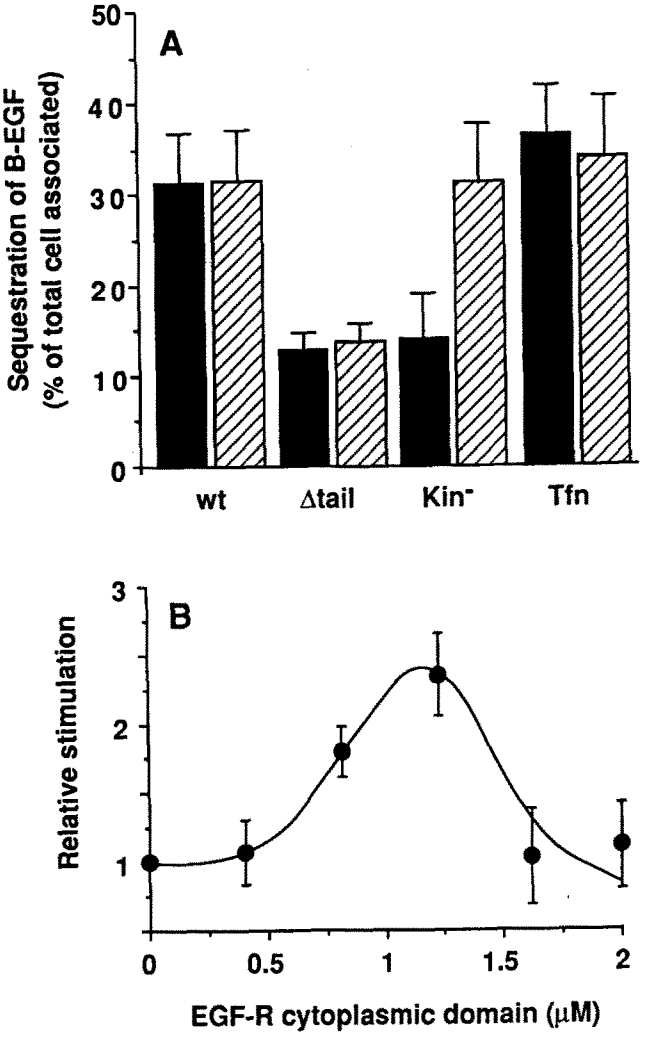

Figure 6. Soluble EGF-R cytoplasmic domain rescues kin^- mutant in trans in a dose-dependent manner. (*A*) Perforated B82L cells expressing either wt or mutant receptors as indicated were assayed for EGF sequestration in the presence (▨) or in absence (■) of cytoplasmic EGF-R cytoplasmic domain (1.2 μM) as described in Fig. 3. The effect on Tfn sequestration in kin^- cells is shown for comparison. The results shown are the average (±SD) of 10 independent experiments utilizing at least three different preparations of purified tyrosine kinase cytoplasmic domain. (*B*) Perforated B82L cells expressing kin^- EGF-R were incubated at 37°C in the presence of increasing amounts of cytoplasmic domain. The results shown are the average (±SD) of three independent titrations and are expressed as the fold-stimulation over the basal rate of sequestration in the absence of added EGF-R cytoplasmic domain.

bound to the Δtail EGF-R mutant corresponded exactly to the low basal rate of sequestration of the antagonist mAb528 (Fig. 4 *B*), confirming that cytoplasmic tail residues are important for efficient ligand-induced recruitment of EGF-R into coated pits (Chang et al., 1993). Significantly, the data in Fig. 3 *C* show that the extent of sequestration of B-EGF by kin^- receptors in perforated cells mirrored the basal rate of sequestration of unoccupied receptors measured using mAb528 as ligand. The basal rate of sequestration of both EGF-R mutants into coated pits was approximately the same as that for unoccupied wt receptors (2–3%/min, Fig. 4, *A–C*, *open symbols*). Only the ligand-induced sequestration of EGF was inhibited in the mutants relative to wt receptors (*closed symbols*). These data demonstrate that the EGF-R kinase activity is specifically required for the ligand-induced recruitment of EGF-R into coated pits.

Autophosphorylation of intact EGF-R is thought to cause a conformational change that exposes internalization sequences on the EGF-R cytoplasmic domain for recognition by the endocytic machinery (Sorkin et al., 1992; Chang et al., 1993). To determine whether the EGF-R itself was the only tyrosine kinase substrate required for ligand-induced endocytosis, we examined EGF sequestration in cells expressing a deletion mutant of the EGF-R lacking the COOH-terminal regulatory domain and all of the autophosphorylation sites required for endocytosis of intact receptors (Walton, 1990; Sorkin et al., 1992). These constructs, referred to as Δkin^+ or Δkin^-, retained a strong endocytic code (Chang et al., 1993) and the intact kinase domain in either the active or inactive ($K^{721}\rightarrow M$ mutant) form (see Fig. 1). The data in Fig. 5 shows that recruitment of the Δkin^+ EGF-R into coated pits occurred with the same efficiency as intact kin^+ receptors. Importantly, this efficient recruitment was entirely dependent on an active tyrosine kinase as EGF was inefficiently sequestered by Δkin^- receptors. These results demonstrate that autophosphorylation of the EGF-R is not sufficient for ligand-induced recruitment of activated EGF-R into coated pits.

Recent experiments have suggested that the recruitment of PI3-kinase to phosphorylated PDGF-receptors might be required for their ligand-induced endocytosis (Joly et al., 1994). Although EGF-R lack the conventional PI-3 kinase-binding sites identified on the PDGF-R, it has been reported that PI-3 kinase activity can be coprecipitated with activated EGF-R (Bjorge et al., 1990). We therefore tested the possibility that PI3-kinase might participate in EGF-R endocytosis by examining the effects of wortmannin, a specific inhibi-

tor of the PI-3 kinase (Yano et al., 1993) on EGF-R recruitment both in vivo and in vitro. Wortmannin, even at high concentrations (100 μM) did not affect the recruitment of EGF-R into coated pits (data not shown), strongly suggesting that PI-3 kinase is not required.

Soluble EGF-R Kinase Domain Rescues kin⁻ Mutant in Trans

We next sought to provide direct evidence for the role of the EGF-R kinase in ligand induced recruitment into coated pits and to establish a functional assay for the identification of the tyrosine kinase substrate(s) required. Thus, a soluble, constitutively active tyrosine kinase derived from the EGF-R (Wedegaertner and Gill, 1989, see Fig. 1) was tested for its ability to restore efficient sequestration of the kin⁻ mutant receptor. The data in Fig. 6 *A* shows that addition of soluble EGF-R tyrosine kinase domain to perforated cells restored the sequestration of kin⁻ receptors to wild-type efficiencies. In contrast, the soluble EGF-R cytoplasmic domain had no effect on either the efficient ligand-induced recruitment of wt receptors or the sequestration of constitutively internalized Tfn-R into coated pits. Similarly, the basal rate of sequestration of Δtail mutant receptors was not affected by addition of soluble kinase reflecting a requirement for other internalization sequences in the EGF-R cytoplasmic domain and confirming that the basal rate of coated vesicle formation was not affected (Chang et al., 1993). These data establish that the restoration of endocytosis was specific to the EGF-R and did not reflect a nonselective stimulation of the endocytic machinery. The two- to threefold kinase-dependent stimulation of kin⁻ EGF-R sequestration corresponded quantitatively to the differences observed between the recruitment of wt and kin⁻ EGF-R in vivo (Fig. 2) and in vitro (Fig. 3 *B*) and also to the differences between basal and ligand-induced rates of sequestration for wt receptors in perforated cells (Fig. 4 *A*). Thus addition of active soluble EGF-R kinase domain in trans to perforated cells appears sufficient to restore efficient ligand-induced sequestration of kin⁻ receptors.

The efficiency of restoration increases with the amount of purified kinase added to perforated B82L cells expressing kin⁻ mutant receptors (Fig. 6 *B*). However, after reaching maximum stimulation at ~1 μM, the ability of soluble EGF-R kinase to stimulate kin⁻ EGF-R recruitment drops to basal levels. The intact cytoplasmic domain of the EGF-R used in these studies not only provides EGF-independent tyrosine kinase activity but also contains the sequences necessary for efficient endocytosis of the EGF-R (Chang et al., 1993). We interpret the decline in stimulation observed at higher concentrations of exogenously added EGF-R cytoplasmic domain to be a consequence of competitive inhibition of soluble internalization codes with those on the intact receptor for limiting factors involved in the recruitment of EGF-R into coated pits. A minimal EGF-R kinase domain lacking internalization codes is being prepared to test this hypothesis.

Discussion

Using a novel cell-free assay that selectively measures early events in endocytosis of EGF-R, we have shown that mutant EGF-R lacking a functional tyrosine kinase cannot undergo efficient recruitment into coated pits. This deficiency can be selectively overcome by supplementing in vitro assays with purified, active soluble EGF-R tyrosine kinase. These results provide the first direct biochemical evidence that an activated EGF-R tyrosine kinase is required for the ligand-induced recruitment of EGF-R to coated pits.

What are the tyrosine kinase substrate(s) required for ligand-induced EGF-R endocytosis? One likely candidate is the EGF-R itself (Helin and Beguinot, 1991; Sorkin et al., 1992). There is a consensus that autophosphorylation of tyrosines located in the regulatory domain of the EGF-R results in a conformational change that removes inhibitory constraints to expose endocytic codes for recognition by the endocytic machinery (Sorkin and Waters, 1993). The finding that each of the internalization codes identified on the EGF-R can function independently as efficient internalization motifs when transplanted onto truncated Tfn-R (Chang et al., 1993) supports this hypothesis.

It is also possible that phosphorylation of the EGF-R might be needed to recruit essential components of the endocytic machinery to the EGF-R either directly or indirectly via SH2 domains. AP2 complexes, a major coat component of plasma membrane-associated coated pits rapidly associate and can be coprecipitated with ligand-activated EGF-R in A431 cells (Sorkin and Carpenter, 1993). Whether this association requires kinase activity and/or autophosphorylation of the EGF-R has not been shown. Similarly, it has recently been shown that PDGF-R lacking the phosphotyrosine-containing binding site for PI-3 kinase, a member of the SH2-protein family, failed to display normal endocytic trafficking (Joly et al., 1994). This is an intriguing result since vps34p, a PI3-kinase analogue is required for protein sorting and transport to the vacuole via a clathrin-dependent pathway in yeast (Hermann et al., 1992). However, we were unable to detect a requirement for PI3-kinase in endocytosis of EGF-R.

Dynamin is another component of the endocytic apparatus that could potentially be recruited to phosphorylated EGF-R since it binds in vitro to the SH3-domain of a number of SH2-domain containing molecules recruited to activated EGF-R in vivo (Gout et al., 1993; Herskovits et al., 1993; Scaife et al., 1994). However, more recent analysis has established that dynamin is a coated pit-associated protein that functions at later stages in the constitutive process of coated vesicle formation and not in the recruitment of receptors into coated pits (Damke et al., 1994). Furthermore, since receptor autophosphorylation is not required for kinase-dependent endocytosis of deletion mutants of the EGF-R it is unlikely that the recruitment of SH2-domain containing proteins to activated EGF-R is essential for endocytosis.

While it seems clear that autophosphorylation of intact EGF-R is necessary for the ligand-induced recruitment of EGF-R into coated pits, we have established that it is not sufficient. Removal of the constraining regulatory domain and all its autophosphorylation sites results in a truncated EGF-R whose recruitment into coated pits is still dependent on an active tyrosine kinase domain. Our in vitro results are entirely consistent with the behavior of these mutant receptors in intact cells (Chang et al., 1991) and suggest that phosphorylation of some other, perhaps cytosolic factor, is required for the efficient recruitment of EGF-R into coated pits. By comparing the biochemical requirements for EGF and Tfn sequestration in perforated A431 cells, we found that ligand-induced recruitment of EGF-R to coated pits spe-

cifically required an as yet unidentified limiting cytosolic factor not required for the constitutive sequestration of Tfn-R (Lamaze et al., 1993). Whether this EGF-R recruiter is a substrate for tyrosine kinase activity is not known.

Receptor-mediated endocytosis of polypeptide growth factors is critical for the attenuation of their cell proliferative signals (Chen et al., 1989; Wells et al., 1990). Studies in intact cells have established that the EGF-R kinase activity is required for receptor down-regulation only at subsaturating concentrations of EGF (Wiley et al., 1991; Felder et al., 1992). Since sub-saturating concentrations of EGF correspond to serum levels, it is intriguing to speculate that the EGF-R recruiter(s) might play an essential physiological role in preventing proliferative signals at sub-threshold levels of EGF-receptor occupancy. In this model, phosphorylation of the EGF-R recruiter by the EGF-activated tyrosine kinase might ensure the rapid and efficient internalization and down-regulation of spuriously activated EGF-receptors. Since EGF is locally synthesized in proliferating tissues, it can accumulate to higher concentrations acting in a paracrine fashion (Murphy et al., 1990). Saturating levels of EGF would overcome the limiting recruiter apparatus so that activated EGF-R might linger at the cell surface able to transmit their mitogenic signals. The phenomenon of saturation of EGF-R endocytosis at higher ligand concentrations has been well-documented in intact cells (Wiley, 1988; Wiley et al., 1991; Felder et al., 1992) and a model in which endocytosis plays a key role in preventing unregulated cell proliferation has been proposed on theoretical grounds (Starbuck and Lauffenberger, 1992). Whether this recruiter is a substrate of the EGF-R tyrosine kinase remains speculative. Identification and isolation of the putative recruiter(s) will clearly be required to test this hypothesis. In the meantime, complementation of kinase-defective EGF-R sequestration in B82L cells using the assay described here, provides a powerful means to identify the tyrosine kinase substrate(s) required for the ligand-induced recruitment of EGF-R into coated pits.

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