Regulated Migration of Epidermal Growth Factor Receptor from Caveolae

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In quiescent fibroblasts, epidermal growth factor (EGF) receptors (EGFR) are initially concentrated in caveolae but rapidly move out of this membrane domain in response to EGF. To better understand the dynamic localization of EGFR to caveolae, we have studied the behavior of wild-type and mutant receptors expressed in cells lacking endogenous EGFR. All of the receptors we examined, including those missing the first 274 amino acids or most of the cytoplasmic tail, were constitutively concentrated in caveolae. By contrast, migration from caveolae required EGF binding, an active receptor kinase domain, and at least one of the five tyrosine residues present in the regulatory domain of the receptor. Movement appears to be modulated by Src kinase, is blocked by activators of protein kinase C, and occurs independently of internalization by clathrin-coated pits. Two mutant receptors previously shown to induce an oncogenic phenotype lack the ability to move from caveolae in response to EGF, suggesting that a prolonged residence in this domain may contribute to abnormal cell behavior.

The most common mutant EGF receptor (EGFR) found in human tumor cells has a truncated extracellular domain, is constitutively active at the cell surface, and is not down regulated by either EGF or anti-EGF IgG (1). Other oncogenic EGFR, those missing the cytoplasmic regulatory domain but having an active kinase domain, also fail to down regulate (2). Down-regulation is the term used to describe the attenuation of signal transduction by receptor-mediated endocytosis. Detailed studies of the normal EGFR have found that endocytosis under these conditions is a high affinity, saturable process that involves the interaction of endocytic codes in the receptor cytoplasmic tail with an unidentified set of molecules present in clathrin-coated pits (3, 4). Receptors that can not down regulate, therefore, are either missing the information required for capture or are incapable of accessing coated pits. None of the studies carried out so far have distinguished between these two mechanisms.

To determine which of these two mechanisms accounts for the behavior of mutant, oncogenic EGFR that fail to down regulate, the location of the unstimulated receptors must first be determined. EGFR might be randomly distributed across the surface or confined to specialized membrane domains. Membrane fractionation and immunocytochemistry has been used to show that in quiescent fibroblasts wild-type EGFR (5, 6), as well as other receptor (7) and non-receptor (8) tyrosine kinases, are highly enriched in caveolae membrane fractions. Moreover, the first phases of signal transduction initiated by EGF binding, such as activation of tyrosine kinase activity (6, 7), phosphorylation of protein substrates (7), recruitment of adaptors (6, 7, 9) and essential kinases (7), and activation of MAP kinase (7, 10), all appear to take place in caveolae membranes. These and other studies (reviewed in Ref. 8) indicate that entire signaling pathways are pre-organized in caveolae. Rapid signal attenuation coincides with loss of receptors from caveolae (6) in response to ligand binding. Depending on the cell type, it takes 3–30 min for EGF to leave caveolae (6, 7). Obviously the exit of the receptor from caveolae must be an important control point in EGFR signal transduction. Spending too little or too much time at this location after ligand binding may be deleterious to the cell.

Mutagenesis has been used to map the critical amino acids in EGFR cytoplasmic tail responsible for both receptor down-regulation and signal transduction. This region of the receptor contains both a kinase and a regulatory domain (11). A mutation in the kinase domain that inactivates kinase activity (M721K) completely abolishes high affinity receptor internalization (12). At least one of the tyrosine residues in the regulatory domain that normally is a substrate for the kinase appears to be required for down-regulation by EGF (13). On the other hand, a constitutively active and tyrosine-phosphorylated receptor that is unable to bind EGF appears not to be spontaneously internalized (14), suggesting separate roles for EGF binding and tyrosine phosphorylation during internalization. Through an analysis of the behavior of truncated receptors, an 18-amino acid sequence between amino acids 973 and 991 was found to be necessary for ligand-stimulated receptor down-regulation and cytosolic calcium increase (3). This region, which consists of a YXXΨ motif flanked by numerous negatively charged amino acids, contains the binding site for clathrin AP2. Unexpectedly, excision of this region from the tail does not affect ligand-induced down-regulation (15). There are two tyrosine residues in the kinase domain (Tyr-891 and Tyr-920) that are not autophosphorylated but are consensus sequences for pp60c-src kinase phosphorylation (16). Activation of G-protein-coupled receptors may stimulate pp60c-src kinase phosphorylation of these sites (17–21). It is not known if the phosphorylation of these residues exerts any control over receptor internalization. Finally, PKC-dependent phosphorylation of Thr-654 blocks ligand-induced down-regulation (4).
Little is known about the mechanism responsible for targeting EGFR to caveolar membrane or what releases the receptor in response to ligand binding. It is possible that those mutations that disrupt EGFr-stimulated internalization prevent migration of the receptor out of caveolae. Mutations that had this effect would provide valuable insight into the mechanism of receptor sequestration by caveolae. For these reasons, we have used previously characterized cell lines expressing wild-type and mutant EGFR to study the plasma membrane distribution of these receptors before and after EGFR stimulation. We find that all of the mutant receptors that are internalization impaired also do not exit from caveolae in response to EGFR. This suggests that internalization of those growth factor receptors that are concentrated in caveolae is a three-step process that involves exit from caveolae, migration in the bulk plasma membrane, and capture by coated pits.

**EXPERIMENTAL PROCEDURES**

**Materials**

Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, iodoxanol, penicillin, G418, and streptomycin were from Life Technologies, Inc. EGF, tyrophostin AG1478, tyrophostin A9, and PFP2 were from Calbiochem (San Diego, CA). Puromycin, tetracycline, endothelin-1, and lysophosphatidic acid (LPA) were from Sigma. Anti- caveolin pAb IgG, anti-Raf-1 mAb IgG, and anti-PKCα mAb IgG were from Transduction Laboratory (Lexington, KY). Anti-phosphorylated Erk pAb IgG was from Promega (Madison, WI), and anti-phosphotyrosine mAb IgG was from Upstate Biotechnology (Lake Placid, NY). Anti-EGFR pAb, agarose-conjugated mAb anti-phosphotyrosine IgG, and anti-erbB2 mAb IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). Carboxyl truncated EGFR were detected using a pAb (designated N13) raised against the 13 amino-terminal acids of EGFR. Horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit IgG were from Cap- pell (Durham, NC). 125I-Protein A, preStanalyzed molecular weight markers, and ECL reagents were from Amersham Pharmacia Biotech. All chemicals for SDS-PAGE electrophoresis were from Bio-Rad. Polyvi- nyldiene difluoride membranes were from Millipore (Bedford, MA).

**Methods**

**Cell Culture**—Rat1B cells and normal human fibroblasts were cultured in DMEM supplemented with 10% v/v fetal bovine serum, 1 mM glutamate, 100 mg/ml penicillin, and 100 µg/ml streptomycin. Cells were grown to near confluence (3–4 days) before removing serum and incubating an additional 24–48 h in the presence of DMEM alone.

The stable HeLa cell lines (HTa) expressing either wild-type or K44A mutant dynamin were generously provided by Dr. Sandra L. Schmid (Department of Cell Biology, Scripps Research Institute, La Jolla, CA). The cells were maintained in DMEM with 10% fetal calf serum in the presence of 200 µg/ml puromycin, 400 µg/ml G418, 2 µg/ml tetracycline, 100 µg/ml penicillin, and 100 µg/ml streptomycin. Sub confluent cultures were harvested and plated at a density of 1.2 × 106 cells/100-mm culture dishes and grown to the presence (uninduced) or absence (induced) of 2 µg/ml tetracycline in DMEM plus 10% fetal calf serum for 48 h before experiments. For the last 24 h, the cells were cultured in the absence of serum.

Stable transfected B22 mouse L cells, which lack endogenous EGFR, expressing either wild-type or mutant human EGFR were grown in DMEM plus 10% fetal calf serum, 80 mM metrotrexate, 100 µg/ml penicillin, and 100 µg/ml streptomycin. Cells were grown to near confluence (2–4 days) and incubated overnight in the presence of DMEM without serum before each experiment.

NR6 cells that lack endogenous EGFR were infected with a retrovi- ruses expressing de-2-7 EGFR, selected in G418 and a cloning line prepared. These cells were maintained in DMEM plus 10% fetal calf serum containing high glucose (45 g/liter) in the presence of 400 µg/ml G418, 100 µg/ml penicillin, and 100 µg/ml streptomycin. Cells were grown to near confluence (2–4 days) and incubated overnight in the presence of DMEM without serum before each experiment.

**Cytosol Acidification and Potassium Depletion**—Human fibroblasts were incubated in DMEM without serum for 24 h before the experiments. To acidify the cytosol, the cells were washed twice with medium A (DMEM plus 20 µM Hepes, pH 7.4) and then incubated in medium A for 30 min at 37 °C. Cells were rinsed twice with medium B (medium A plus 10 mM acetic acid, pH 5.0) and incubated in medium B for 10 min at 37 °C. Acidified cells were washed twice in phosphate-buffered saline and incubated in the absence or presence of EGF (50 ng/ml) for the indicated times in DMEM. Intracellular potassium depletion (22) was carried out by rinsing cells twice with medium A and incubating them in the same medium for 30 or 60 min. Cells were rinsed with buffer A (50 mM Tricine-HCl, pH 7.5, 1 mM EDTA, 250 mM NaCl, 1% Triton X-100, and 100 µM NaCl) and incubated in hypotonic buffer A (buffer A diluted 1:1 with water) for 5 min at 37 °C. The cells were rinsed three times in buffer A and incubated in the same buffer for 30 min at 37 °C, washed twice with phosphate-buffered saline before adding EGF (50 ng/ml) and incubating for the indicated times in DMEM.

**Caveolae Isolation**—Caveolae were isolated by the detergent-free method of Smart et al. (5). The cells were centrifuged at 53,000 × g for 90 min, the postnuclear supernatant was pooled and desiganted as non-caveolae membrane. The top 5 ml of the gradient was mixed with 4 ml of buffer C, overlaid with 1 ml of 15% w/v iodoxanol in buffer B, followed by 0.5 ml of 5% w/v iodoxanol in buffer B. The gradients were centrifuged at 53,000 × g for 90 min, and caveolae were collected from the 5%/15% interface (0.5 ml).

**SDS-PAGE and Western Blotting**—Protein concentrations were determined by the method of Bradford (Bio-Rad) using bovine serum albumin as a standard. Samples were dissolved in SDS-PAGE sample buffer (62 mM Tris, pH 8, 0.5% w/v SDS, 10% glycerol, 0.5% w/v bromphenol blue), loaded on a 4% stacking gel, and separated using a 5–15% linear gradient gel. Proteins were transferred to polyvinylidene difluoride membrane by electrophoretic transfer at 50 V for 2 h on ice. After membranes were incubated with buffer D (20 mM Tris, pH 7.6, 137 mM NaCl) plus 5% nonfat dry milk and 0.5% Tween 20 for 1 h at room temperature. The membranes were then incubated with primary antibodies diluted in buffer D plus 0.1% nonfat dry milk and 0.2% Tween 20 for 2 h at room temperature. The membranes were washed with buffer D plus 0.2% milk and 0.2% Tween 20 for 15 min and twice for 5 min at room temperature, and then incubated with the appropriate horserad- ish peroxidase-conjugated anti-IgG antibody in 0.1% bovine serum albumin in buffer D plus 1% milk and 0.2% Tween 20 for 1 h. The membranes were then washed once for 15 min and four times for 5 min each with buffer D plus 0.2% milk and 0.2% Tween 20. Staining was detected using enhanced chemiluminescence (ECL). Apparent molecular masses were estimated using prestained molecular weight markers (broad range). To measure the relative amounts of EGFR in each fraction, the horseradish perox- idase-conjugated goat anti-IgG antibody was detected using ECL. A. Gels were incubated in the presence of 1 µCi/ml 125I-protein A for 1 h. The membranes were then washed once for 15 min and four times for 5 min each with buffer D plus 0.2% milk and 0.2% Tween 20. The radioactive intensity of the EGF receptor band (180 kDa) was measured using a Molecular Dynamics PhosphorImager (Sunnyvale, CA).

**Immunoprecipitation**—The cells were washed in ice-cold buffer B and collected by scraping in the same buffer. Cells were lysed by Dounce homogenization in buffer B containing protease inhibitors (1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml benzamidine, 1 µg/ml soybean trypsin inhibitor) and phosphatase inhibitors (0.5 mM sodium vanadate, 10 mM sodium pyrophosphate, and 10 mM sodium fluoride). A postnuclear supernatant fraction was prepared by spinning the lysate at 90 × g for 10 min. The postnuclear supernatant was layered over 23 ml of ice-cold 50% Percoll in buffer B. After centrifugation at 85,000 × g for 30 min, the cytosol and plasma membrane fractions were collected. The plasma membrane fraction was briefly sonicated (six 50-Joule bursts at 10 watts each), mixed with buffer C (50% w/v iodoxanol in buffer B plus 40 µl sucrose) to a final iodoxanol concentration of 23% and overlaid with 6 ml of linear (10–20%) gradient of iodoxanol in buffer B. Samples were centrifuged at 53,000 × g for 90 min. The samples were washed once for 15 min and then mixed with 6 ml of linear (10–20%) gradient of iodixanol in buffer B. The samples were washed once for 15 min and then mixed with 6 ml of linear (10–20%) gradient of iodixanol in buffer B. The gradients were centrifuged at 53,000 × g for 90 min, and caveolae were collected from the 5%/15% interface (0.5 ml).

**Other Methods**—Each experimental results presented is a representa- tive example from three to five trials using identical conditions.

Acidified cells were washed twice in phosphate-buffered saline and incubated in the absence or presence of EGF (50 ng/ml) for the indicated times in DMEM. Intracellular potassium depletion (22) was carried out by rinsing cells twice with medium A and incubating them in the same medium for 30 min at 37 °C. Cells were rinsed twice with medium B (medium A plus 10 mM NaCl) and incubated in medium B for 10 min at 37 °C.
**RESULTS**

**Regulation of EGFR Loss from Caveolae**—Previously, we reported that EGFR receptors are concentrated in caveolae membrane fractions isolated from human fibroblasts (5). We used quantitative immunoblotting to estimate the percentage of total surface EGFR receptors that were in the caveolae fraction of these cells before and after incubation in the presence of EGF (Table I, HF). Plasma membranes from quiescent fibroblasts were used to prepare caveolae and non-caveolae fractions. Immunoblots of each fraction showed that we recovered most of the plasma membrane EGFR receptors in the two fractions and that 60.5% of these receptors were in the caveolae fraction. EGFR was enriched 11.6-fold in the caveolae fraction relative to the whole membrane. After exposure to EGF, there was a marked decline in the total number of receptors in the plasma membrane and a decrease in the percentage of these receptors in the caveolae fraction.

Previously, we have used Rat-1 cells to study the dynamics of EGFR behavior (6). Like the human fibroblast, the caveolae fraction of these cells was enriched in EGFR (Table I, Rat-1). In quiescent cells, 48.6% of the receptors were in the caveolae fraction (Rat-1 - EGF) and they were enriched 18-fold. After 20 min in the presence of EGF, the caveolae fraction contained only 14% of the remaining EGFR. To determine if receptor loss was coupled to internalization by clathrin-coated pits, we used immunoblotting to look at the effects of cytosol acidification and potassium depletion, two treatments that inhibit receptor-mediated endocytosis (22, 23), on EGFR migration from caveolae (Fig. 1). In untreated human fibroblasts, EGFR was concentrated in the caveolae fraction (A, lane 1). The receptor in this fraction markedly declined after a 20-min incubation in the presence of EGF at 37 °C (compare lanes 1 and 2). Caveolin-1, by contrast, did not change during the incubation. Neither cytosol acidification (lanes 3 and 4) nor potassium depletion (lanes 5 and 6) prevented loss of EGFR from the caveolae fraction. Moreover, incubation of cells in the presence of EGF at 4 °C (lanes 7 and 8), conditions that block uptake by coated pits (24), had little effect on receptor loss from caveolae.

Similar results were obtained with HeLa cells expressing K44A dynamin (Fig. 1B), a dominant-negative acting mutant dynamin I that blocks internalization of EGFR by coated pits (25). Caveolae fractions were prepared from cells expressing either wild-type (WT) or K44A (K44A) mutant dynamin in the absence of 2 µg/ml tetracycline for 48 h to stimulate expression of the respective protein. The cells were cultured in the absence of serum for 24 h before being incubated in the presence of 100 ng/ml EGF for 0, 2, 20, or 60 min at 37 °C. Caveolae fractions (CM) were isolated, separated by gel electrophoresis (10 µg/lane) and immunoblotted with either anti-EGFR IgG (EGFR) or anti-caveolin-1 IgG (Caveolin). C, serum-starved, normal human fibroblasts were either not treated (lanes 7 and 8) or incubated in the presence of 10 µM tyrphostin AG1478 (lanes 1 and 2), 10 µM PP2 (lanes 3 and 4), or 10 µM tyrphostin A9 (lanes 5 and 6) for 10 min before EGF was added to the dish and the cells incubated further for the indicated time. Caveolae fractions were isolated and immunoblotted.

**FIG. 1. Effects of inhibiting receptor-mediated endocytosis** (A and B) and tyrosine kinase activity (C) **on** migration of EGFR from caveolae. A, serum-starved, normal human fibroblasts were either not treated (lanes 1 and 2) or subjected to cytosol acidification (lanes 3 and 4), potassium depletion (lanes 5 and 6), or 4 °C (lanes 7, 8). Each set of cells was then washed and incubated for 0 or 20 min in the presence of 50 ng/ml EGF at either 37 °C (lanes 1–6) or 4 °C (lanes 7 and 8). Caveolae fractions were prepared and separated by gel electrophoresis (10 µg/lane) using 5–15% gradient gels and immunoblotted with either anti-EGFR IgG (EGFR) or anti-caveolin-1 IgG (Caveolin). B, stable HeLa cell lines expressing either wild-type (WT) or K44A (K44A) mutant dynamin were incubated in the absence of 2 µg/ml tetracycline for 48 h to stimulate expression of the respective protein. The cells were cultured in the absence of serum for 24 h before being incubated in the presence of 100 ng/ml EGF for 0, 2, 20, or 60 min at 37 °C. Caveolae fractions (CM) were isolated, separated by gel electrophoresis (10 µg/lane) and immunoblotted with either anti-EGFR IgG (EGFR) or anti-caveolin-1 IgG (Caveolin). C, serum-starved, normal human fibroblasts were either not treated (lanes 7 and 8) or incubated in the presence of 10 µM tyrphostin AG1478 (lanes 1 and 2), 10 µM PP2 (lanes 3 and 4), or 10 µM tyrphostin A9 (lanes 5 and 6) for 10 min before EGF was added to the dish and the cells incubated further for the indicated time. Caveolae fractions were isolated and immunoblotted.
EGFR prevent receptor internalization by coated pits (12). We used various tyrosine kinase inhibitors to determine if kinase activity was required for receptor migration out of caveolae (Fig. 1C). Tyrophostin AG1478 preferentially inhibits EGFR kinase (26). The presence of this inhibitor markedly blocked the ability of EGF to stimulate receptor migration out of the caveolae fraction (compare lanes 1 and 2 with lanes 7 and 8). We next looked at inhibitors that are more specific for Src kinases (PP2; Ref. 27) and the platelet-derived growth factor receptor kinase (tyrophostin A9; Ref. 28). The Src kinase inhibitor PP2 partially blocked EGFR migration compared with AG1478 (compare lanes 3 and 4 with lanes 7 and 8). By contrast, tyrophostin A9 had no effect on migration (compare lanes 5 and 6 with lanes 7 and 8).

Phorbol 12-myristate 13-acetate (PMA) stimulates PKC phosphorylation of EGFR at threonine 654 and blocks EGFR-induced, high affinity internalization of the receptor (4). To determine if PMA has any effect on EGFR loss from caveolae, we preincubated normal human fibroblasts in the presence or absence of 100 nM PMA for 10 min and then added EGF for various times (Fig. 2). Caveolae were isolated and immunoblotted with antibodies to either EGFR or caveolin-1 (A) or caveolin-1 IgG (Caveolin). B, B82 cells transfected with either T654A mutant (T654A) or wild-type (WT) EGFR were incubated in the presence (lanes 1–6) or absence (lanes 7–12) of 100 nM PMA for 10 min before adding 100 ng/ml EGF to the dish (lanes 2, 3, 5, 6, 8, 9, 11, and 12) and incubated further for the indicated time. Caveolae fractions were prepared and separated by gel electrophoresis (10 µg/lane) using 5–15% gradient gels and immunoblotted either with anti-EGFR IgG (EGFR) or anti-caveolin-1 IgG (Caveolin).
this fraction and immunoblotted with anti-EGFR IgG. Low amounts of EGFR were present in immunoprecipitates from untreated cells (lane 1) but markedly increased within 3 min after exposure to EGF (lane 2). The concentration of phosphorylated receptors increased further after 20 min but declined by 60 min. Both endothelin ET-1 (Et-1) and LPA (LPA) also stimulated the appearance of EGFR in the anti-PY IgG immunoprecipitates (compare lanes 5–7 and lanes 8–10). We reproducibly found that the kinetics of receptor phosphorylation were different for LPA than for endothelin ET-1 (compare lanes 5–7 with lanes 8–10).

Cells incubated in the presence of EGF rapidly lost EGFR from the caveolae membrane fraction (Fig. 3B; EGFR, lanes 1–4), which was accompanied by a transient increase in the concentration of phosphorylated Erk1 and Erk2 (pMAPK, lanes 1–4) in this fraction. By contrast, neither endothelin ET-1 (EGFR, lanes 5–7) nor LPA (EGFR, lanes 8–10) induced EGFR loss from the caveolae fraction. Each of these ligands did, however, stimulate activation of caveolae MAP kinase (pMAPK, lanes 5–10), an indication that EGFR in this fraction was activated. MAP kinase activation occurred rapidly and was sustained when endothelin ET-1 was added to the media (lanes 5–7) while there was a significant lag before LPA activated MAP kinase. LPA also reproducibly induced the appearance of higher amounts of activated MAP kinase than did the other ligands. Interestingly, whereas LPA and EGF stimulated Raf-1 recruitment to caveolae, endothelin ET-1 did not (data not shown). These results indicate that all three ligands stimulate EGFR phosphorylation (A) but only EGF causes receptor migration out of caveolae (B).

**Mutant EGFR Impaired in Migration from Caveolae**—The presence of EGFR in caveolae must be regulated through interactions between the receptor and specific molecular elements within the caveolae membrane. So far the results suggest that two sets of interactions are necessary. One set specifies the localization of receptors to caveolae fraction. The other controls receptor migration to a different membrane domain in response to specific stimuli. We examined the behavior of mutant receptors to try and identify the critical parts of the EGFR necessary for both localization and migration.

We used either B82 mouse fibroblasts or NR6 cells transfected with cDNAs coding for various mutant EGFR. Neither of these cells express endogenous EGFR, but after transfection each cell expressed comparable numbers of the indicated receptor. B82 cells expressing wild-type cDNA have 10-fold more receptors than Rat-1 cells (31). Quantitative immunoblotting showed that the caveolae fraction contained 42% of these receptors and they were enriched 6.7-fold (Table I, B82).

Cells were incubated in the presence of EGF for various times before isolating caveolae and assaying for the presence of EGFR by immunoblotting (Fig. 4). All the receptors we tested were concentrated in the caveolae membrane fraction before EGF was added to the media (lane 1). After the addition of EGF, however, we identified two classes of receptors: those that immediately disappeared from the caveolae fraction and those retained for the time course of the experiment (lanes 1–3). The migratory receptors included the wild-type, T654A, c958f993–1186, c958f1022–1186, c958f991–1022, and c1022. By contrast, M721K, c991, c958, c688, c647, and de 2–7 were all retained in caveolae after addition of EGF. The comparative behavior of these mutant receptors indicates that migration of receptors out of caveolae membrane requires both the auto-phosphorylation of at least one of the five tyrosine residues in the receptor regulatory domain and the binding of EGF.

As is the case for normal human fibroblasts (7) and Rat-1 cells (9), the highest concentration of plasma membrane-associated Erk1 and Erk2 in B82 and NR6 cells was in the caveolae fraction (data not shown). All of the mutant receptors that contained functional kinase domains (wild-type, T654A, c958f993–1186, c958f1022–1186, c958f991–1022, and c1022, c991, and c958) were able to stimulate the activation of these MAP kinases in response to EGF (lanes 4 and 5). In cells expressing de 2–7, however, the same amount of activated MAP kinase was present in the caveolae fractions regardless of whether the cells had been exposed to EGF (de 2–7, lanes 4 and 5). This is consistent with reports that this mutant receptor has a basal tyrosine kinase activity and does not bind EGF (32, 33). We also monitored Raf-1 recruitment to the caveolae fraction in cells expressing wild-type and mutant receptors. In cells expressing receptors that contained a portion of the regulatory domain, EGF stimulated the recruitment of Raf-1 to the caveolae fraction (Fig. 4, lanes 6 and 7). Receptors containing active kinase domains but lacking the regulatory domain also stimulate Raf-1 recruitment (c991 and c958, lanes 6 and 7). Interestingly, even though the concentration of c958 and c991 (lanes 1–3) did not decline, recruitment of Raf-1 to caveolae...
Internalization-defective EGFR in Caveolae

A normal human fibroblasts were serum-starved for 24 h and incubated in the presence of 50 ng/ml EGF for 0, 2, 20, and 60 min at 37 °C. Caveolae fractions (lanes 1–4) and non-caveolae fractions (lanes 5–8) were isolated, separated by gel electrophoresis (5 μg/lane), and immunoblotted with antibodies to EGFR (EGFR), anti-ErbB2 IgG (ErbB2), or anti-caveolin-1 IgG (Caveolin). B, B82 cells transfected with the wild-type (lanes 1–4), kinase-negative M721K (lanes 5–8), c’958 truncated (lanes 9–12), or c’991 truncated (lanes 13–16) EGFR were serum-starved for 24 h before incubating in the presence of 100 ng/ml EGF for 0, 2, 20, or 60 min at 37 °C. Caveolae fractions were isolated, separated by gel electrophoresis (10 μg/lane), and immunoblotted with antibodies to EGFR (EGFR), anti-ErbB2 IgG (ErbB2), or anti-caveolin-1 IgG (Caveolin). C, B82 cells transfected with the wild-type (lanes 1–4), kinase-negative M721K (lanes 5–8), c’958 truncated (lanes 9–12), or c’991 truncated (lanes 13–16) EGFR were serum-starved for 24 h before incubating in the presence of 100 ng/ml EGF for 0, 2, 20, or 60 min at 37 °C. At the end of the incubations, postnuclear supernatant fractions were prepared and phosphotyrosine-containing proteins were immunoprecipitated. Each precipitate was separated by gel electrophoresis and immunoblotted with anti-ErbB2 IgG (pErbB2).

membrane was transient (data not shown). Receptors lacking kinase activity (M721K and c’647, lanes 6 and 7) did not stimulate Raf-1 recruitment.

Normal and Mutant EGFR Activate ErbB2—EGFR lacking the phosphotyrosine sites for recruiting SHC and Grb2 stimulate downstream signaling as effectively as wild-type receptors (34), which is consistent with our observation that the binding of EGF to c’958 very effectively stimulated MAP kinase activation in caveolae (Fig. 4). One possibility is that the truncated receptor forms a heterodimer with ErbB2 (35, 36) and activates MAP kinase through this intermediate. We used immunoblotting to study the dynamics of ErbB2 localization to the caveolae membrane fraction (Fig. 5). Caveolae (A, lanes 1–4) and non-caveolae (A, lanes 5–8) membrane fractions isolated from quiescent normal human fibroblasts were separated by gel electrophoresis (equal protein loads) and immunoblotted with anti-EGFR IgG (A, EGFR), anti-ErbB2 IgG (A, ErbB2), or anti-caveolin-1 IgG (A, Caveolin). Both EGFR and ErbB2 were highly enriched in caveolae fractions (compare caveolae to non-caveolae). The addition of EGF to media stimulated the rapid loss of EGFR from the caveolae fraction (caveolae) but had only a modest effect on the level of ErbB2 in this fraction. Even after a 60-min incubation in the presence of EGF, the concentration of ErbB2 in the caveolae fraction remained high. Therefore, even though ErbB2 and EGFR appear to be in close physical proximity in caveolae, EGFR preferentially migrates to non-caveolae membrane in response to EGF.

We next looked at the effects of EGF on the distribution of ErbB2 in cells expressing truncated receptors (Fig. 5B). EGF stimulated the loss of the wild-type receptor (EGFR) from caveolae fraction but the concentration of ErbB2 remained relatively unchanged (WT, compare EGFR with ErbB2). EGF did not stimulate the loss of kinase-negative receptors (M721K) from the caveolae fraction, and the amount of ErbB2 in this fraction did not decline. Likewise, even though EGF did not stimulate the loss of the kinase-active, truncated receptors (c’958 and c’991 from the caveolae fraction, the concentration of ErbB2 remained unchanged (B, compare EGFR with ErbB2 in c’958 and c’991). These results suggest that truncated receptors may remain in close proximity to ErbB2 for extended periods of time after cells are exposed to EGF.

A potential measure of how long EGFR interacts with ErbB2 is the length of time ErbB2 remains phosphorylated after EGF binds. Cells transfected with wild-type, M721K, c’958, or c’991 receptors were starved of serum for 24 h before they were incubated in the presence of EGF for various times (Fig. 5C). At the end of each incubation, tyrosine-phosphorylated proteins were immunoprecipitated from whole cell lysates, separated by gel electrophoresis, and immunoblotted with anti-ErbB2 IgG mAb. The amount of ErbB2 immunoprecipitated from wild-type EGFR cells increased between 0 and 20 min of EGF stimulation but declined by 60 min (WT), which paralleled the loss of EGFR from the caveolae fraction (B, WT). EGF did not stimulate phosphorylation of ErbB2 in cells expressing kinase-negative receptors (M721K). By contrast, the amount of phosphorylated ErbB2 in cells expressing either c’958 or c’991 receptors (c’958, c’991) was as high at 60 min as at 20 min (compare lanes 11, 12, 15, and 16 with lanes 3 and 4).

DISCUSSION

Regulation of Receptor Migration—All of the normal and mutant EGFR we tested were found to be concentrated in the caveolae membrane fraction of quiescent cells. This included c’647, which only has a 11-amino acid cytoplasmic tail, and de 2–7, which is missing amino acids 6–274 of the extracellular domain. Therefore, the information required for targeting the receptor to caveolae membrane is contained within amino acid 275–647 of the receptor. This rules out a role for the putative caveolin-1 binding motif (37) in receptor clustering and focuses attention on the possibility that the transmembrane domain targets growth factor receptors to caveolae membrane.

The rapid exit of EGFR appears to require autophosphorylation of at least one of the five tyrosine residues in the regulatory domain of the receptor. Kinase-negative receptors (M721K) did not migrate (Fig. 4) while attaching as few as 31 amino acids of the regulatory domain (amino acids 991–1022) to the carboxyl end of an active kinase domain (at amino acid 958) was sufficient. This construct (c’958 991–1022) lacks most of the actin binding region in the receptor (amino acids 984–996; Ref. 38), so actin does not play a direct role in receptor exit from caveolae. Even though endothelin ET-1 and LPA stimulated EGFR phosphorylation, this population of phosphorylated receptors did not migrate out of caveolae (Fig. 3). Pre-
vious studies indicate that both cytokines can stimulate the Src-dependent phosphorylation of EGFR (19, 20) and that Tyr-891 and Tyr-920 in the kinase domain of the receptor are consensus sites for Src phosphorylation (16). Therefore, these two tyrosine residues, which are in the kinase domain, may play an additional role in controlling receptor traffic out of caveolae. The ability of the inhibitor PP2 to partially block receptor exit from caveolae indicates a role for Src family kinases, too. This is in agreement with the finding that overexpression of Src kinase stimulates an increase in the rate of EGFR endocytosis (39). Finally, most likely EGF binding is also required to get receptors to move out of caveolae because de 2–7, which does not bind EGF (32, 33), was concentrated in caveolae even though it has a constitutively active kinase domain (see Fig. 4).

With the exception of c'991, all of the mutant receptors that are internalization-impaired also do not disappear from the caveolae membrane fraction in response to EGF binding. Moreover, both PMA and tyrosine kinase inhibitors, which are known to prevent coated pit-mediated internalization of EGF (31), blocked exit. In the case of c'991, we incubated cells in the presence of EGF for up to 60 min (Fig. 4) but still saw little loss of receptor from the caveolae fraction. Previous studies have shown that internalization of c'991 receptors is markedly slower than wild-type receptors (13), with only ~50% internalization by 60 min. One explanation for our findings is that only those c'991 receptors present in non-caveolae membranes are initially internalized whereas those in caveolae exit very slowly.

PMA blocked the loss of EGFR from the caveolae fraction, possibly by inhibiting receptor tyrosine kinase activity (4). By contrast, the migration of full-length receptors bearing a T654A substitution was not affected by PMA. PMA causes T654A receptors to have a lower affinity for EGF than wild-type receptors do in the absence of PMA (4). Likewise, EGFR in cells expressing K44A dynamin have a low affinity for EGF (40). The fact that both populations of receptors migrated out of caveolae membrane with normal kinetics suggests that the affinity of EGF for EGFR has little influence on the exit of the receptor from caveolae. Finally, migration from caveolae and internalization by coated pits appears not to be linked because four independent methods of inhibiting receptor-mediated endocytosis (4 °C, K + depletion, cytosol acidification, and K44A dynamin) had no effect on EGF-stimulated receptor loss from caveolae fractions.

EGFR Molecular Interactions in Caveolae—An EGFR truncated at the COOH terminus of the kinase domain (c'958) was just as effective as wild-type receptor at stimulating the phosphorylation of the Erk1 and Erk2 in caveolae fraction (Fig. 4). EGFR form heterodimers with other members of the ErbB family (35, 36, 41) and previous studies have shown that truncated EGFR (c'973) can signal through ErbB2 (34). Since truncated EGFR appear not to migrate out of caveolae in response to EGF, we were curious how they would be able to interact with ErbB2 if the two receptors were located in different regions of the plasma membrane. Our results suggest, however, that both truncated EGFR and ErbB2 are concentrated together in caveolae membranes. We cannot be sure that both types of receptors are in the same caveola, but these results raise the possibility that a common targeting motif causes them both to accumulate in the same membrane domain. The close proximity of the two receptors in caveolae might facilitate heterodimer formation, which would facilitate the transfer of information laterally through complimentary signaling pathways (35).

In contrast to EGFR, the concentration of ErbB2 in caveolae only declined slightly in response to EGF, which suggests that interactions between these two signaling pathways may be restricted to caveolae membranes. The inefficient movement of ErbB2 out of caveolae may be related to its impaired internalization by clathrin-coated pits (42). Activation of two different types of truncated receptors (c'958 and c'991) did not cause any loss of ErbB2. The prolonged presence of both truncated EGFR and ErbB2 in caveolae after EGF binding would result in persistent signal transduction through the ErbB2 pathway. Indeed, the EGF-stimulated tyrosine phosphorylation pattern of ErbB2 was markedly prolonged for truncated receptors (Fig. 5C, c'958 and c'991) compared with wild-type receptors (Fig. 5C, W7).

EGF signal transmission is initiated by the combinatorial interactions between receptor and multiple molecular intermediates. Each set of interactions is dictated by: (a) the local concentration of the collaborating molecules and (b) the molecular ecology of the surrounding membrane. The current study, together with previous work, indicates that EGFR can be located in at least three different membrane compartments after EGF binds; caveolae, bulk plasma membrane, and clathrin-coated pits. It is possible, therefore, that interactions occurring in caveolae may induce one set of signaling events while another set of signals is broadcast once the receptor reaches clathrin-coated pits. Through this type of receptor behavior, signal transduction becomes compartmentalized at the cell surface, thereby allowing a single class of receptors to transmit different signals from distinct locations in the cell. For example, if EGFR can only interact with ErbB2 when the two are in caveolae, then a critical determinant in EGF signaling through ErbB2 is whether or not EGFR is in caveolae at a time when EGF is bound.

It should be possible to identify the signaling pathways that emanate from each of the various compartments visited by a migratory receptor by pinpointing where EGF stimulates the combinatorial interactions required for the initiation of a specific signaling event. For example, we have shown previously that EGF stimulates the recruitment and activation of Raf-1 in caveolae (6), which is linked to the activation of a resident population of MAP kinase (7). Therefore, activation of the entire MAP kinase pathway can occur in caveolae, even in vitro (10). In the current study, we show that both LPA and endothelin-ET-1 stimulate the phosphorylation of those EGFR that appear to be concentrated in caveolae (Fig. 3), suggesting that lateral signaling between these two pathways can occur in this compartment. We also found that kinase-active de 2–7 was constitutively located in caveolae, and recent evidence indicates that this mutant EGFR causes the continuous activation of both c-Jun N-terminal kinase (43) and phosphatidylinositol 3-kinase (1). Caveolae may be the compartment where pathways dependent on the activation of these molecules originate. On the other hand, kinase-active receptors that are unable to move out of caveolae (c'991) cannot stimulate cell motility (44). The COOH-terminal addition of regulatory domain tyrosines to these receptors renders them competent to stimulate cell movement and to exit from caveolae. The signaling pathways in control of cell migration, therefore, may originate from receptors that are not in caveolae. We also showed in the current study that PMA prevents movement of wild-type receptor from caveolae but not T654A mutant receptors. The ability of PMA to inhibit EGF-stimulated mitogenesis is lost in cells expressing T654Y mutant receptors (45). This raises the possibility that EGFR control mitogenesis from a non-caveolae compartment. We must stress that these examples merely indicate that caveolae have the capacity to initiate certain signaling path-
ways. More work is needed to determine if they are an exclusive site.

**Mislocalized Receptors Are Oncogenic**—An important conclusion of this study is that abolishing high affinity internalization of EGFR also impairs the ability of the receptor to migrate out of caveolae. Numerous studies have documented that multiple EGFR signaling intermediates are concentrated in the caveolae fraction (8). The prolonged residence of kinase-active receptors of caveolae, therefore, offers the potential for an unregulated stimulation of those signaling pathways that use these intermediates. Indeed, two (c'958 and de 2–7) of the three mutant kinase-active receptors that were impaired in leaving caveolae have previously been found to be oncogenic (2, 46, 47). Our study suggests that the oncogenic phenotype of cells expressing these receptors may be caused by the inability of these receptors to migrate out of caveolae membrane. If this is true, moving these mutant receptors out of caveolae should allow the cells to revert to a normal phenotype.

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