A Leucine-based Determinant in the Epidermal Growth Factor Receptor Juxtamembrane Domain Is Required for the Efficient Transport of Ligand-Receptor Complexes to Lysosomes*

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Ligand binding causes the epidermal growth factor (EGF) receptor to undergo accelerated internalization with eventual degradation in lysosomes. The goal of this study was to investigate the molecular basis of endocytic sorting, focusing on post-internalization events. We have identified a sequence located between amino acid residues 675 and 697, encompassing a dileucine motif at residues 679 and 680, that enhances endosome-to-lysosome transport when conformational restraints in the EGF receptor carboxyl terminus are removed by truncation. The same dileucine motif is also necessary for efficient lysosomal transport of ligand-occupied full-length EGF receptors. A L679A,L680A substitution diminished the degradation of occupied full-length EGF receptors without affecting internalization but had a significant effect on recycling. Rapid recycling of mutant receptors resulted in reduced intracellular retention of occupied EGF receptors and delayed down-regulation of cell surface receptors. We propose that the L679A,L680A substitution acts primarily to impair transport of ligand-receptor complexes through an early endosomal compartment, diverting occupied receptors to a recycling compartment at the expense of incorporation into lysosome transport vesicles. We also found that mutant receptors with truncations at the distal half of tyrosine kinase domain (residues 809–857) were not efficiently delivered to the cell surface but were destroyed in an endoplasmic reticulum-associated degradative pathway.

Epidermal growth factor (EGF) and related polypeptides elicit biological responses through binding to specific cell surface receptors belonging to the ErbB family of protein-tyrosine kinases (reviewed in Ref. 1). EGF regulates the intracellular trafficking of ligand-EGF receptor (EGFR) complexes, causing accelerated internalization from clathrin-coated pits, retention in endosomes, and ultimately degradation in lysosomes (reviewed in Ref. 2). Ligand-regulated EGFR down-regulation therefore modulates cellular responses to growth factors by controlling the duration of signaling from the cell surface and by transporting activated EGFRs to intracellular compartments where they continue to signal (reviewed in Ref. 3). In addition, because EGFR is the only ErbB receptor that undergoes ligand-induced down-regulation (4), signal transduction by different members of the ErbB family may be regulated by compartmentalization in the endocytic pathway. The importance of EGFR endocytic transport to normal proliferation is exemplified by the fact that EGFRs that fail to internalize have been associated with cell transformation (5) and tumorigenesis (6).

EGFR down-regulation is a complex process regulated by many factors, including ligand occupancy, receptor aggregation, tyrosine kinase activity, endosomal acidification, and intrinsic sorting signals (2). It is also clear that the multiple transport steps in the endocytic pathway are facilitated by different molecular interactions (7–10). Internalization is regulated by endocytic codes located in the EGFR carboxyl-terminal domain that are functionally interchangeable with the internalization signal of the transferrin receptor (11). Internalization also involves interactions between cytoplasmic sequences in activated EGFRs and plasma membrane clathrin AP-2 adaptor proteins (7, 11–15). Although a tyrosine-based signal encompassing carboxyl-terminal domain residues 973–977 mediates AP-2 binding in vitro, the physiological relevance of this interaction is unclear, because EGFRs with an internal deletion of this region undergo normal ligand-induced internalization (16). Interestingly, AP-2 also interacts with two EGFR substrates: eps15, which is constitutively associated with AP-2 in vivo (17), and SHC, which binds AP-2 in vitro (18). Another signaling intermediate required for efficient EGFR internalization is Grb2, which binds to activated EGFRs either directly or as part of a SHC-Grb2 complex (1). It has been proposed that Grb2 provides a phosphoinositide-dependent link to dynamin (19, 20), a GTPase that regulates endocytosis (16, 21). Taken together, these studies indicate that EGFR internalization from clathrin-coated pits is facilitated by multiple interactions occurring simultaneously.

Following internalization, ligand-receptor complexes are diverted from a recycling pathway to lysosomes (10). Endosome-to-lysosome sorting presumably involves the signal-mediated transfer of ligand-receptor complexes to vesicles called endosomal carrier vesicles or multivesicular bodies (ECMVBs), which transport material to late endosomes (reviewed in Ref. 22). Although candidate sorting sequences have been identified in the carboxyl half of the cytoplasmic domain, a consensus has not been reached regarding the structural and enzymatic requirements for transporting EGFRs to lysosomes (9, 23, 24).
One candidate lysosomal sorting molecule, SNX-1, has been identified that binds at the distal border of the tyrosine kinase domain (see Fig. 1 and Ref. 25).

The goal of this study was to further characterize the molecular basis for selective transport of ligand-EGFR complexes to lysosomes. This was accomplished by analyzing a series of EGFR proteins with progressive truncations encompassing the entire cytoplasmic domain to test the hypothesis that cryptic sorting sequences normally masked in unoccupied full-length EGFRs would be exposed in truncated receptors. In contrast to previous studies, we analyzed sequences in the juxtamembrane domain as well as the carboxyl terminus for two reasons. First, the EGFR cytoplasmic domain has numerous consensus leucine-based signals implicated in lysosomal targeting of a number of membrane proteins (26–29) located throughout the cytoplasmic domain. Second, the juxtamembrane domain contains a sorting signal that regulates EGFR basolateral delivery in polarized Madin-Darby canine kidney cells (30), suggesting that this region may have a broader role in vesicular transport.

We have found that a sequence located between amino acid residues 675 and 697 in the cytoplasmic juxtamembrane region enhances endosome-to-lysosome transport of truncated EGFRs. Residues Leu679 and Leu680, which conform to a leucine-based sorting signal, were shown to be a critical determinant for efficient lysosomal transport of cytoplasmically truncated EGFRs. This same motif was also required for the efficient transport of ligand occupied full-length EGFR complexes to lysosomes.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis**—EGFR cytoplasmic truncation and substitution mutants were made using polymerase chain reaction (PCR) to modify EGFR coding sequences cloned in pCB6, a eukaryotic expression plasmid containing a human cytomegalovirus regulatory region, transcription termination and polyadenylation signals from the human growth hormone gene, an SV40 origin of replication and early region promoter-enhancer, and a neomycin resistance gene (reviewed in Ref. 31). To create cDNAs encoding receptors with cytoplasmic truncations, stop codon substitutions were made at codons for Pro716, Ala719, Val811, Pro812, Gly813, Tyr814, Leu815, and Val821. Forward primers (listed below) were designed to anneal to sequences 5′ to pCB6 compatible with the pCB6 mutagenic primers (listed below) were designed to create a premature enzyme site (in parentheses) in the EGFR coding sequence. Reverse mutagenic primers (listed below) were designed to create a premature stop codon (in bold) 400–500 nucleotides downstream of the forward primer, as well as a restriction site (underlined and in parentheses) compatible with the pCB6 polymerase. PCR fragments were gel-purified and incorporated into plasmid DNA by phosphorylation and restriction enzyme digestion. The PCR products were ligated into pCB6′/EGF+/ DVD) per ml of methionine- and cysteine-free minimal essential medium supplemented with 10% dialyzed fetal bovine serum and 0.2% BSA. In some experiments, labeling medium was replaced with chase medium consisting of serum-free DMEM supplemented with nonradioactive methionine (0.75 mg/ml) and cysteine (1.2 mg/ml), and cells were incubated for an additional period of time before harvesting. Cells were labeled with 50 Ci of Express Protein Labeling mix (1175 Ci/mmol; New England Nuclear Research Products, Wilmington, DE) per ml of methionine- and cysteine-free minimal essential medium supplemented with 10% dialyzed fetal bovine serum and 0.2% BSA. For Western blotting, proteins resolved by SDS-PAGE were transferred to nitrocellulose according to standard procedure (38). For cell labeling, cells were trypsinized, lysed with TRIzol reagent (Life Technologies, Inc.) for 5 min at room temperature. The chemical cross-linker was quenched by a 5-min incubation in a solution of 0.1 M HEPES, pH 7.4, supplemented with 0.12 M NaCl, 5 mM MgSO4, 0.2 mM phenylmethanesulfonyl fluoride, and 1 μM leupeptin. EGFRs were immuno precipitated with EGFR-I adsorbed onto protein A-Sepharose CL-4B beads (Sigma). Samples were solubilized with Laemmli buffer and separated by SDS-polyacrylamide gel electrophoresis (PAGE) (37). Gels were treated with En3Hance (New England Nuclear) for fluorography.

**Cell Labeling, Immunoprecipitation, and Western Blots**—Cells were rinsed twice with methionine- and cysteine-free minimal essential medium and then labeled with 50 μCi of Express Protein Labeling mix (1175 Ci/mmol; New England Nuclear Research Products, Wilmington, DE) per ml of methionine- and cysteine-free minimal essential medium supplemented with 10% dialyzed fetal bovine serum and 0.2% BSA. For Western blotting, proteins resolved by SDS-PAGE were transferred to nitrocellulose according to standard procedure (38). EGFRs were detected by immunoblotting with an affinity purified rabbit polyclonal antibody specific to amino acids 1005–1016 in the EGFR cytoplasmic domain (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Phosphorytosing-containing proteins were detected by immunoblotting with an anti-phosphotyrosine-horseradish peroxidase conjugate (Transduction Laboratories, Lexington, KY).

**1-EGF Cross-linking**—Receptor grade mouse EGF (Toyobo Biochemicals, Osaka, Japan) was labeled with 125I (carrier-free, >350 mCi/μl; New England Nuclear) using chloramine-T. Cells were rinsed three times with ice-cold DMEM supplemented with 0.2% BSA and then incubated with approximately 10 μg 125I-EGF for 2 h at 4°C. Cells were rinsed again with the DMEM/BSA solution to remove unbound ligand and then incubated with 2 μM disuccinimidyl suberate (Pierce) on a rotisserie shaker at 37°C for 2 h. The reaction was terminated by the addition of 0.1 M HEPES, pH 7.4, supplemented with 0.12 M NaCl, 0.2 mM CaCl2, 0.5 mM MgCl2, 80% ethanol, and water, for 15 min at room temperature. The chemical cross-linker was quenched by a 5-min incubation with 0.05 M Tris, pH 7.4, at room temperature. Cell lysates were prepared using 1% Nonidet P-40 exactly as described above, and total cell protein was separated by SDS-PAGE.

**Northern Blots**—Cells were trypsinized, lysed with TRIzol reagent (Life Technologies, Inc.) for 5 min at room temperature, and then...
extracted with chloroform:isoamyl alcohol (24:1 v/v). RNA was precipitated with isopropanol, washed with 70% ethanol, and resuspended in distilled H₂O treated with 0.01% diethylpyrocarbonate. 20 µg of total RNA was denatured, fractionated by electrophoresis in a 1.4% agarose/formaldehyde gel, and transferred to Biotrans (+) nylon membrane (ICN Biomed, Costa Mesa, CA) using standard techniques (39). An oligonucleotide complementary to nucleotides 2227–2247 in the human EGFR cDNA was labeled with [α-32P]dCTP (3000 Ci/mmol; Amersham Pharmacia Biotech) using Klenow reagent and a Random Primed DNA Labeling kit from Boehringer-Mannheim. Blots were prehybridized for 2 h at 65 °C with a solution containing 1 µg EDTA, 250 mM sodium phosphate, pH 7.2, 7% SDS, 1% BSA, and 50 µg/ml salmon sperm DNA. Blots were hybridized with 1 × 10⁶ cpm/µl of 32P-labeled oligonucleotide in the same solution for 18 h at 65 °C. Blots were washed three times with a solution of 20 mM sodium phosphate, pH 7.5, 1 µg EDTA, and 10% BSA at 65 °C, air-dried, and exposed to film for autoradiography.

Treatment with Brefeldin A—Cells were preincubated in medium supplemented with vehicle (1 µl of MeOH/ml) or brefeldin A (BFA) (5 µg/ml) 30 min, and then incubated in nonradioactive chase medium for periods up to 2 h before lysis with Nonidet P-40. Labeling and chase media were also supplemented with vehicle or BFA.

Digestion with Endoglycosidase H—Pulse-labeled EGF receptors collected by immunoprecipitation were solubilized with 50 µl of a solution of 1% SDS and 5% β-mercaptoethanol in 0.1 M sodium citrate, pH 5.5. 20–µl aliquots were incubated with an equal volume of distilled H₂O or with 1 × 10⁶ cpm/µl of 32P-labeled oligonucleotide for 18 h at 37 °C with 1 milliunits endoglycosidase H (endo H; 40 units/mg enzyme; Boehringer-Mannheim). Equal aliquots were subject to sham digestion.

Internalization Assays—Cells were seeded at a density of 5 × 10⁶ cells/well in six-well tissue culture plates 48 h before each assay and refed with DMEM supplemented with 25 mM HEPEs and 0.2% BSA (D/H/B) 4 h prior to the assay. Cells were rinsed with cold D/H/B, and incubated with 250 µg/ml of 125I-labeled 528-Fab or 1–100 ng/ml of 125I-labeled EGF diluted in the same medium for 2 h at 4 °C. The EGF-specific 528-Fab Fc recognizes an extracellular peptide epitope and is widely used, for example to track intracellular transport of unoccupied EGF receptors (24). 528-Fabs (gift of Starla Blick, Dept. of Pediatrics, Case Western Reserve University) were iodinated exactly as described above. Cells were warmed to 37 °C for periods up to 20 min and then incubated with 100 ng/ml of nonradioactive EGF for periods up to 2 h. Cells were rinsed two times with ice-cold D/H/B and then incubated with 100 ng/ml 125I-EGF for 1 h at 4 °C. Surface-bound 125I-EGF was removed by incubating cells with acid stripping buffer (pH 2.8) for 2.5 min on ice, and radioactivity was determined by γ counting. To measure EGF receptor down-regulation, cells were preincubated with 100 ng/ml nonradioactive EGF for periods up to 2 h. Cells were rinsed two times with D/H/B and then incubated with 100 ng/ml 125I-EGF for 1 h at 4 °C. Surface-bound 125I-EGF was removed by incubating cells with acid stripping buffer (pH 2.8) for 2.5 min on ice, and radioactivity was determined by γ counting. To measure EGF receptor internalization, cells were preincubated with 100 ng/ml nonradioactive EGF for 24 h later. Cells were rinsed twice with cold D/H/B and then incubated with 100 ng/ml 125I-EGF for 1 h at 4 °C. Surface-bound 125I-EGF was removed by incubating cells with acid stripping buffer (pH 2.8) for 2.5 min on ice, and radioactivity was determined by γ counting. 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the proteins shown in transfected with cDNAs encoding each of codons. Locations of a dileucine motif coded by cDNAs with premature stop cytoplasmic domains for wild-type EGFR main (7), and conserved NPXY motifs (25) are also shown. B and C, COS-1 cells transfected with cDNAs encoding each of the proteins shown in A were assayed for EGFR expression 48 h post-transfection. B, cells were metabolically labeled for 4 h, lysed with Nonidet P-40, and immunoprecipitated with an EGFR mAb specific for an extracellular epitope common to monkey and human EGFRs. C, intact cells were chemically cross-linked with \(^{125}\)I-EGF. Immunoprecipitates (B) or total cellular protein (C) were separated on 7.5% SDS-PAGE gels. Locations of full-length 170-kDa and cytoplasmically truncated EGFRs are indicated on the right. Molar mass standards: myosin, 200 kDa; \(\beta\)-galactosidase, 116.3 kDa; phosphorylase B, 97.4 kDa. Jx, juxtamembrane.

Fig. 2. Metabolic turnover of EGFRs with cytoplasmic truncations. COS-1 cells transfected with EGFR cDNAs shown in Fig. 1A were metabolically labeled for 3 h starting at 48 h post-transfection. Labeling medium was replaced with chase medium, and cells were lysed with Nonidet P-40, and immunoprecipitated with a receptor-specific mAb, and immunoprecipitates were separated on 7.5% SDS-PAGE gels. Locations of full-length 170-kDa and truncated EGFRs are indicated on the left. WT, wild type; Jx, juxtamembrane.

either time point. EGFR proteins with truncations in the carboxyl-terminal domain exhibited stability similar to full-length EGFRs, as did c'-674 and c'-651 receptor proteins with truncations in the juxtamembrane domain. However, stability of two proteins truncated near the proximal border of the kinase domain, c'-697 and c'-723, was markedly reduced compared with endogenous monkey EGFRs. Analysis of EGFRs with cytoplasmic truncations therefore identified two classes of receptors with reduced protein expression: those that failed to undergo efficient transport to the cell surface (c'-809, c'-885, and c'-957) and those that were transported to the cell surface but had reduced stability compared with wild-type EGFRs under basal conditions (c'-697 and c'-723).

Reduced Stability of EGFR Proteins with Cytoplasmic Truncations Is Mediated by Two Distinct Post-translational Mechanisms—Based on results in Fig. 1, we hypothesized that EGFRs with truncations in the distal kinase domain (c'-809, c'-885, and c'-957) were degraded in the biosynthetic pathway. To test that hypothesis, we first showed that cDNAs encoding these truncated EGFRs produced stable mRNAs as judged by Northern blot analysis (Fig. 3A). We then asked whether the fungal metabolite BFA affected protein turnover, because the endoplasmic reticulum (ER)-associated degradative pathway is BFA-insensitive (40). Transfected COS-1 cells that had undergone a mock-treatment or a preincubation with BFA were pulse-labeled for 45 min and then incubated in chase medium for periods up to 2 h (Fig. 3B). BFA treatment had no effect on turnover of c'-809 receptors (Fig. 3B) or c'-885 receptors (not shown) and little effect on turnover of c'-957 receptor (Fig. 3B). The molecular weight of endogenous EGFRs was reduced in BFA-treated cells, consistent with BFA-induced blockade of Golgi-mediated carbohydrate processing. These data suggest that although a fraction of c'-957 receptors appear to be degraded in a post-Golgi compartment, receptors with truncations in the distal half of the kinase domain are mostly disposed of by a BFA-insensitive, ER-associated degradative pathway (41).

Similar to truncated receptors such as c'-973 (Fig. 3C), which exhibits normal stability, c'-697 (Fig. 3C) and c'-723 (not shown) receptors were not subject to BFA-insensitive, ER-associated degradation. In addition, c'-697 and c'-723 receptors were transported through the Golgi complex with approximately the same kinetics as wild-type EGFRs. This was shown using endo H digestion to distinguish mature EGFRs containing a mixture of complex and high mannose-type N-linked oligosaccharides from EGFR precursors containing only high mannose N-linked oligosaccharides (36, 42). Because endo H only cleaves high mannose oligosaccharides (43), endo H resist-
Figure 3. Reduced stability of EGFR proteins with cytoplasmic truncations is mediated by two distinct mechanisms. A. Northern blot analysis of human EGFR mRNA expression. 20 μg of total RNA from cells transfected with cDNAs encoding truncated EGFRs listed in the figure were fractionated in 1.4% agarose/formaldehyde gels and transferred to nitrocellulose. Blots were incubated with a 32P-labeled oligonucleotide probe complementary to nucleotides 2227–2247 in the human EGFR cDNA. Sizes of transcripts are indicated beneath each lane. B and C, effect of BFA on degradation of EGFRs with cytoplasmic truncations. COS-1 cells transfected with cDNAs encoding c'-957 or c'-809 EGFRs (B), or c'-973 or c'-697 EGFRs (C), received a mock treatment (1 μl of MeOH/ml) or were preincubated with BFA (5 μg/ml) for 1 h, pulse-labeled for 30 min, and then incubated in chase medium for times indicated before lysis with Nonidet P-40. Labeling and chase media were also supplemented with vehicle or BFA. Cell lysates were immunoprecipitated with a receptor-specific mAb, and immunoprecipitates were separated on 7.5% SDS-PAGE gels. WT, wild type; kb, kilobases.

Figure 4. Acquisition of endo H resistance by EGFRs with cytoplasmic truncations involving juxtamembrane or proximal kinase domains. COS-1 cells transfected with cDNAs encoding EGFR proteins listed in the figure were pulse-labeled for 20 min starting at 45 h post-transfection. Labeling medium was replaced with chase medium for times indicated. Cells were lysed with Nonidet P-40 and immunoprecipitated with a receptor-specific mAb. Half of each immunoprecipitate was subjected to a mock digestion (-Endo H), and the remainder was incubated for 18 h at 37 °C with 1 milliunit of endo H (+Endo H). Immunoprecipitates were separated on 7.5% SDS-PAGE gels. P, deglycosylated precursor protein; M, mature protein.

Role for Leu679-Leu680 in c'-697 Receptor Degradation—To understand the molecular basis of the enhanced turnover of c'-697 and c'-723 receptors, we examined the amino acid sequence of EGFR residues 675–697 to identify potential consensus sorting signals (Fig. 6). Residues Leu679 and Leu680 were of particular interest because leucine-based motifs are known to regulate lysosomal trafficking of several membrane proteins (reviewed in Ref. 45). Furthermore, Leu679-Leu680 and adjacent amino acids (residues 679–683) are identical to sequences at the carboxyl terminus of an adenovirus early region 3 membrane protein called E3–13.7 (46). Because E3–13.7 re-routes the carboxyl terminus of an adenovirus early region 3 membrane protein called E3–13.7 (46). Because E3–13.7 re-routes from a recycling pathway to lysosomes.
To test the hypothesis that a Leu679-Leu680-based sorting signal is utilized during ligand-induced transport, we expressed the L679A,L680A substitution in the context of a full-length EGFR (EGFR/L679A,L680A). As shown in Fig. 8A, permanent NR6 cell lines transfected with plasmids encoding either wild-type EGFR or EGFR/L679A,L680A were incubated with or without 100 ng/ml EGF for 15 min and lysed with Nonidet P-40. Cell lysates were immunoprecipitated (IP) with EGF-R1. Immunoprecipitates were transferred to nitrocellulose and immunoblotted (IB) using a second EGFR-specific antibody directed against a carboxyl-terminal epitope. Cell lysates were immunoprecipitated with a biotin-conjugated phosphotyrosine (PY) antibody. Immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with an horseradish peroxidase-conjugated phosphotyrosine antibody.

Western blotting using a phosphotyrosine-specific antibody. As shown in Fig. 8B, EGF stimulation induced tyrosine phosphorylation of both proteins to a similar extent and also caused a molecular weight shift reflecting the increase in tyrosine phosphorylation. These data indicate that the L679A,L680A substitution does not interfere with ligand-induced EGFR activation.

Two approaches were used to determine the effect of L679A,L680A substitution on ligand-induced EGFR down-regulation. First, NR6 cells expressing either wild-type EGFR or EGFR/L679A,L680A were assayed for steady-state 125I-EGF binding following a preincubation with unlabeled EGF for periods up to 2 h. Down-regulation of wild-type EGFRs reached a plateau of 60% in cells that had been preincubated with 1 ng/ml ligand for 1 h and remained at the same level with longer preincubations (Fig. 9). In contrast, EGFR/L679A,L680A receptors were down-regulated by less than 20% after 1 h of preincubation with unlabeled ligand and continued to undergo down-regulation at later time points (Fig. 9). The second approach for analyzing ligand-induced down-regulation was to measure EGFR half-lives. Cells that had been incubated in chase medium for 3 h after a 45-min pulse-label were stimulated with ligand for periods up to 2 h, and cell lysates were immunoprecipitated using an EGFR-specific mAb (Fig. 10). As shown in Fig. 10, the pool of radiolabeled receptors was relatively

**Fig. 6.** Amino acid sequence and predicted structure of EGFR residues 675–697. A, organization of EGFR cytoplasmic domain and amino acid sequence of a putative signal encompassing Leu679-Leu680. Also shown are two other putative lysosomal sorting signals: residues 945 and 957 encompassing the binding site for SNX-1 in the kinase catalytic core domain (25) and residues 1022–1123 (Ly) in the carboxy-terminal domain (23). B, amino-terminal post-translational phosphorylation sites (mitogen-activated protein kinase sites Thr669 and Ser671) and terminal domain (23). Amino-terminal post-translational phosphorylation sites are characteristic of other known consensus leucine-based motifs. An amphipathic helix predicted for this region is highlighted by cross-hatched bar. B, amphipathic helical representation of residues 675–697, with charged residues (±) aligned on one side of the helix and hydrophobic residues (in italics) on the other side. The L679A,L680A substitution is also indicated.

**Fig. 7.** Metabolic half-lives of cytoplasmically truncated EGFRs. Cells were metabolically labeled for 3 h starting at 48 h post-transfection, changed to chase medium, and lysed with Nonidet P-40 at indicated times. Cell lysates were immunoprecipitated with a receptor-specific mAb, and immunoprecipitates were separated on 7.5% SDS-PAGE gels. Radiolabeled endogenous EGFRs and EGFRs with cytoplasmic truncations (top) were quantitated by phosphorstorage autoradiography (bottom). Data are presented as the percentage of radiolabeling at the 3-h chase time point.

**Fig. 8.** Expression of full-length wild-type EGFR or EGFR/L679A,L680A in NR6 cells. Permanent NR6 cells expressing wild-type EGFR or EGFR/L679A,L680A were incubated with or without 100 ng/ml EGF for 15 min and lysed with Nonidet P-40. A, cell lysates were immunoprecipitated (IP) with EGF-R1. Immunoprecipitates were transferred to nitrocellulose and immunoblotted (IB) using a second EGFR-specific antibody directed against a carboxyl-terminal epitope. B, cell lysates were immunoprecipitated with a biotin-conjugated phosphotyrosine (PY) antibody. Immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with an horse radish peroxidase-conjugated phosphotyrosine antibody.
The Role of L679A,L680A on Internalization of Full-length EGFRs—We next asked whether the inability of ligand to down-regulate EGFR/L679A,680A as efficiently as wild-type EGFR was associated with differences in internalization. To measure basal internalization, cells were incubated with 125I-EGF (100 ng/ml) for periods up to 2 h. Cell lysates were immunoprecipitated with a receptor-specific mAb, and immunoprecipitates were separated on 7.5% SDS-PAGE gels. Interestingly, we consistently observed an increased amount of radioactivity associated with EGFR/L679A,680A at the 60-min time point, suggesting that these molecules may have enhanced solubility in Nonidet P-40. These data therefore indicate that the mutant EGFRs are degraded less efficiently than wild-type EGFRs following ligand occupation.

To test the hypothesis that Leu 679-Leu680 is part of a signal for selective transport of ligand-EGFR complexes to lysosomes, we examined cytoplasmically truncated EGFRs exhibiting reduced protein stability, we identified two categories of receptor proteins with enhanced degradation phenotypes: those exhibiting enhanced degradation after reaching the cell surface and those undergoing rapid degradation shortly after biosynthesis. In addition, we demonstrated that a dileucine motif in the EGFR juxtamembrane domain is required for efficient lysosomal transport of truncated EGFRs as well as ligand occupied full-length EGFRs.

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rates (panels A and B, respectively. Data for deriving ligand-induced $K_{in}$ under saturating conditions (100 ng/ml) are not shown. Ligand-induced recycling rates ($K_{rec}$) were calculated by linear regression analysis of data from Fig. 12. Ligand-induced EGFR half-lives ($t_{1/2}$) were calculated by linear regression analysis of values derived from PhosphorImager quantitation of data in Fig. 10.

TABLE I

| Wild-type EGFR | EGFR/L679A,L680A |
|----------------|------------------|
| Basal $K_{in}$ (% min$^{-1}$) | 1.50 | 1.50 |
| Ligand-induced $K_{in}$ (% min$^{-1}$) | 6.40 | 5.69 |
| 1 ng of EGF/ml | 4.55 | 5.92 |
| 100 ng of EGF/ml | 2.72 | 4.52 |
| Ligand-induced $K_{rec}$ (% min$^{-1}$) | 1.9 | 3.5 |
| Ligand-induced EGFR $t_{1/2}$ (h) | |

who have shown that the EGFR juxtamembrane domain is not important for ligand-induced receptor uptake (7, 11).

Although wild-type and mutant receptors facilitated the rapid accumulation of intracellular ligand, steady-state levels of accumulated ligand were substantially lower in cells expressing the mutant EGFR than in cells expressing wild-type EGFR. The reduced ability of cells expressing mutant receptors to accumulate intracellular ligand was also evident in down-regulation assays monitoring the disappearance of cell surface ligand binding sites. Additionally, mutant EGFRs were not as efficiently transported to lysosomes as wild-type EGFRs, as shown by differences in the biosynthetic half-lives of ligand-occupied EGFRs. EGFR/L679A,L680A receptors did, however, exhibit faster recycling kinetics than wild-type EGFRs. These data suggest that reduced ligand accumulation and down-regulation exhibited by EGFR/L679A,L680A receptors is due to enhanced recycling of internalized receptors from endosomes.

A closer examination of the amino acid sequence surrounding Leu$^{678}$-Leu$^{680}$ reveals that this region conforms to other known leucine-based signals by several criteria. For example, the targeting activity of leucine-based motifs is often influenced by an acidic residue four or five residues amino-terminal to the leucine pair (49). In addition, leucine-based signals may be regulated by post-translational modification, because they often have nearby phosphorylation sites (45). The putative signal encompassing Leu$^{678}$-Leu$^{680}$ has both an appropriately distanced acidic residue (Glu$^{673}$) as well as nearby mitogen-activated protein kinase phosphorylation sites (Thr$^{669}$ and Ser$^{671}$) (Fig. 6).

Although leucine-based signals implicated in lysosomal transport were originally identified in membrane proteins with relatively short (20–30 amino acids) cytoplasmic tails, critical leucine motifs also have been found in juxtamembrane regions of other membrane proteins with more extensive cytoplasmic domains, such as the T-cell receptor CD3 $\gamma$ subunit (29) and the insulin receptor (50).

The altered transport of the EGFR/L679A,L680A is probably involved in the regulation of EGFR-mediated cell signaling events.

Fig. 11. Internalization of EGFR-specific $^{125}$I-Fab or $^{125}$I-EGF by NR6 cell lines expressing human EGFRs. NR6 cells were preincubated for 2 h at 4 °C with either EGFR-specific $^{125}$I-Fabs (250 ng/ml) (A) or $^{125}$I-labeled EGF (1 ng/ml) (B) and then switched to 37 °C for times indicated in the figure. After removing surface-bound Fab or ligand by acid-stripping, cells were solubilized with 1 N NaOH to determine internalized radioactivity. Internalization is represented as the percentage of total radioactivity associated with the interior of the cell. The values are the means ± S.D. (A, n = 6; B, n = 6). Some standard error bars are obscured by symbols. WT, wild type.

Fig. 12. $^{125}$I-EGF recycling kinetics in NR6 cell lines expressing full-length human EGFRs. NR6 cells were preincubated for 2 h at 4 °C with $^{125}$I-labeled EGF (1 ng/ml) and then switched to 37 °C for 5 min to allow for internalization. After removing surface-bound ligand by mild acid-stripping, $^{125}$I-EGF-loaded cells were incubated with a 100-fold excess of unlabeled EGF for 1 h at 4 °C and then incubated at 37 °C for the indicated periods of time. At the end of each incubation, media were collected to determine the amount of intact and degraded $^{125}$I-EGF as described under ‘‘Experimental Procedures.’’ Surface bound $^{125}$I-EGF was removed by a harsh acid strip, and cells were solubilized with 1 N NaOH to determine cell-associated radioactivity. The sum of intact $^{125}$I-EGF in the media and surface-bound $^{125}$I-EGF released by a harsh acid strip was expressed as the percentage of total radioactivity in the media and the cells at each time point. The values are the means ± S.D. (n = 6). Some standard error bars are obscured by symbols. WT, wild type.
EGF Receptor Sorting to Lysosomes

In summary, our results indicate that the efficient transport of ligand-receptor complexes to lysosomes depends on an intrinsic signal encompassing Leu<sup>679</sup>-Leu<sup>680</sup> located at EGFJR juxtamembrane domain. The Leu<sup>679</sup>-Leu<sup>680</sup> signal exerts its effect by facilitating movement of occupied EGFJRs through an early endocytic compartment. Along with other studies that have identified a basolateral sorting signal in this same region (30), we suggest that the EGFJR juxtamembrane domain may have a broad role in vesicular transport.

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