The Epidermal Growth Factor Receptor Juxtamembrane Domain Has Multiple Basolateral Plasma Membrane Localization Determinants, Including a Dominant Signal with a Polyproline Core*

Cheng He‡, Michael Hobert‡‡, Leslie Friend‡, and Cathleen Carlin‡§**

From the ‡Department of Physiology and Biophysics, School of Medicine, Case Western Reserve University, the ¶Case Western Reserve University Cancer Center, and the §The Rainbow Center for Childhood PKD, Rainbow Babies and Children’s Hospital of the University Hospitals of Cleveland, Cleveland, Ohio 44106

The epidermal growth factor (EGF) receptor is located predominantly in the basolateral membrane of polarized epithelia, where it plays a pivotal role during organogenesis and tissue homeostasis. We have previously found that a 22-amino acid sequence in the EGF receptor juxtamembrane domain contains autonomous sorting information necessary for basolateral localization using the Madin-Darby canine kidney epithelial cell model. The goal of this study was to determine the molecular basis of EGF receptor basolateral membrane expression using site-directed mutagenesis to modify specific residues in this region. We now show that this sequence has two different, functionally redundant basolateral sorting signals with distinct amino acid requirements: one dependent on residues 658LL659 conforming to well-characterized leucine-based sorting signals, and a second containing a polyproline core comprising residues Pro667 and Pro670 (667XXP670). Our data also suggest that Arg662 contributes to the function of the proline-based signal. 667XXP670 was the dominant signal when both motifs were present and was more effective than 658LL659 at overriding strong apical sorting signals located in the same molecule. Site-directed mutations at Arg662, Pro667, and Pro670 were also associated with increased apical expression of full-length EGF receptors, demonstrating for the first time that the juxtamembrane region is necessary for accurate polarized expression of the native molecule.

The ability to establish and maintain plasma membrane asymmetry is a fundamental property of polarized epithelial cells that form physical barriers between various body compartments (reviewed in Refs. 1 and 2). Epithelial cell plasma membranes are organized into distinct apical and basolateral compartments (reviewed in Refs. 1 and 27). Many different epithelial cell types (reviewed in Refs. 1 and 28–30) have been implicated in basolateral transport and compartmentalized cell signaling (1–3). The dynamic nature of plasma membrane asymmetry allows for epithelial cell plasticity and the ability to respond to a variety of physiological cues. Alterations in membrane polarity are often associated with epithelial cell dysfunction and pathophysiology, stressing the central role of spatial organization in normal cell function (reviewed in Ref. 4).

Epithelial membrane asymmetry is due in part to signal-mediated domain-selective membrane protein sorting from intracellular compartments (reviewed in Refs. 5–7). A number of relatively diverse apical sorting signals have now been identified, including glycosphosphatidylinositol (GPI) membrane anchors (8, 9), ectodomain glycans (10, 11), and transmembrane or ectodomain amino acid sequences (12). Basolateral sorting signals are generally composed of relatively short cytoplasmic amino acid sequences (6, 7), and many are related to tyrosine (13, 14) or leucine (15, 16)-based signals originally identified as clathrin-coated membrane localization signals (reviewed in Refs. 17 and 18). Tyrosine- and leucine-based signals are linked to clathrin via interactions with specific clathrin adaptor protein (AP) subunits (reviewed in Refs. 19 and 20). The three major classes of mammalian APs are AP-1 found predominantly at the trans-Golgi network (TGN), AP-2 at the plasma membrane, and AP-3 at the TGN and endosomes (19, 21, 22). Although a role for AP-facilitated transport in clathrin-dependent pathways is well-established (19), it is only recently that these molecules have been implicated in basolateral transport at the TGN (23, 24). Other basolateral sorting signals have been identified, however, that bear no relation to known clathrin-coated membrane localization motifs. Other than clusters of essential charged amino acids, most of the signals in this category are devoid of recognizable motifs that might offer clues regarding physiologically relevant protein-protein interactions (13, 25, 26).

The EGF receptor is located in the basolateral membrane in many different epithelial cell types (reviewed in Refs. 1 and 27). Nowhere is the importance of restricted basolateral EGF receptor expression more apparent than in polycystic kidney disease (28–30), where apically mislocalized receptors have a major role in disease progression (31, 32). We have shown

* This work was supported in part by a March of Dimes Birth Defects Foundation grant and by National Institutes of Health (NIH) Grant P50 DK54178 (to C. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by NIH Training Grant T32-HL07717. Current address: Dept. of Pathology, Emory University School of Medicine, 1639 Pierce Dr., Atlanta, GA 30322.

‡ To whom correspondence should be addressed: Dept. of Physiology and Biophysics, School of Medicine, 10900 Euclid Ave., Cleveland, OH 44106-4970. Tel.: 216-368-8939; Fax: 216-368-5586; E-mail: cxc39@email.ohio-state.edu.

The abbreviations used are: GPI, glycosphosphatidylinositol; AP, adaptor protein; TGN, trans-Golgi network; EGF, epidermal growth factor; EGFR, EGF receptor; MDCK, Madin-Darby canine kidney; MEM, minimal essential medium; FBS, fetal bovine serum; CHO, Chinese hamster ovary; CMV, cytomegalovirus; FITC, fluorescein isothiocyanate; CLSM, confocal laser scanning microscopy; DAF, decay accelerating factor.

1 The abbreviations used are: GPI, glycosphosphatidylinositol; AP, adaptor protein; TGN, trans-Golgi network; EGF, epidermal growth factor; EGFR, EGF receptor; MDCK, Madin-Darby canine kidney; MEM, minimal essential medium; FBS, fetal bovine serum; CHO, Chinese hamster ovary; CMV, cytomegalovirus; FITC, fluorescein isothiocyanate; CLSM, confocal laser scanning microscopy; DAF, decay accelerating factor.
Basalateral EGF Receptor Expression

previously that newly synthesized EGF receptors are delivered directly to the basolateral surface in the MDCK cell model (33) and have identified sorting information located between juxtamembrane domain residues Lys652 and Ala674 necessary for domain-specific targeting of cytoplasmically truncated receptors (26) (see Fig. 1A). Importantly, this same sequence mediates basolateral targeting when transplanted to a heterologous reporter molecule, proof that it is an autonomous, dominant signal (26). Residues Lys652 through Ala674 lack critical tyrosine residues (26) and do not overlap any of the EGF receptor sorting signals necessary for clathrin-mediated internalization located in the carboxyl terminus (34). In addition to MDCK cells, EGF receptors also exhibit a predominantly basolateral localization in LLC-PK1 cells (35, 36), which lack a novel epithelial cell-specific AP-1 subunit isoform necessary for correctly sorting basolateral membrane cargo with AP-dependent sorting signals (37). Hence, polarized EGF receptor sorting from internal compartments is likely mediated by novel protein-protein interactions. Data presented in this study show that the EGF receptor juxtamembrane domain has a hierarchy of functionally redundant basolateral membrane localization signals with distinct amino acid requirements.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Madin-Darby canine kidney (MDCK) epithelial cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 2% glutamine. MDCK cells were seeded on polycarbonate Transwell filter inserts (0.4-μm pore size) (Costar Corp., Cambridge, MA) at a density of 5 × 10^5 cells per 12-mm filter, or 5 × 10^6 cells per 75-mm filter, to generate electrically resistant monolayers with well-developed tight junctions suitable for domain-specific assays 4–6 days later (26, 33). Chinese hamster ovary (CHO) cells were maintained in the alpha formulation of MEM supplemented with 10% FBS and 2% glutamine.

**Mutagenesis and Chimeric cDNAs**—Procedures for replacing EGF receptor residues Lys656, Leu662, and Pro667 with premature stop codons followed by a restriction site compatible with the polynuclein in the eukaryotic expression plasmid pcB6* have been described previously (26, 33). Cytoplasmically truncated receptors are named based on the carboxy-terminal amino acid in the EGF receptor-coding region (e.g. c-674). The c-674 receptor was made using PCR and a full-length EGF receptor filter or 5/H11003 monolayers with well-developed tight junctions suitable for domain-specific assays. To make permanent MDCK cell lines, cells seeded at a density of 10^6 cells per 75-mm filter, to generate electrically resistant monolayers with well-developed tight junctions suitable for domain-specific assays 4–6 days later (26, 33). Chinese hamster ovary (CHO) cells were maintained in the alpha formulation of MEM supplemented with 10% FBS and 2% glutamine.

** point mutations were introduced into the full-length EGF receptor using a cDNA template cloned in pBK-CMVlacDIII. pBK-CMVlacDIII is a derivative of a pBK-CMV phagemid (Stratagene Cloning Systems, La Jolla, CA), created by deleting nucleotides 1098–1299 in the inducible lac promoter (38) and by eliminating a unique DraIII site at nucleotide 240 in the fl (fl origin of replication. Neither the EGF receptor nor the reporter molecule, proof that it is an autonomous, dominant signal (38). Sequences were amplified using the same forward primer described in the previous paragraph and one of the following reverse mutagenic primers designed to introduce a specific amino acid substitution (bold italics), a DraIII site (underlined) to facilitate subcloning, and a silent SacI site (double underlined) to facilitate recombination writing with a specific amino acid sequence: R662T, 5/CCTCGAGTTATATGAGCACTCCAGAAGC-3; S668P, 5/GGCTCTAGATGAGCACTCCAGAAGC-3; T670I, 5/GGCGTTAGATGAGCACTCCAGAAGC-3; and P670L, 5/GGCGTTATCTGGTGTCGCTCCAGAAGC-3. PCR products were digested at an EcoNI site located at EGF receptor nucleotide 1876 and the DraIII site located at the 5'-end of the PCR products, and ligated to full-length EGF receptor sequences cloned in pBK-CMVlacDIII digested with the same enzymes. When sequenced, the recombinant products had an internal deletion, due to a second previously unrecognized EcoNI site located 5' to the nucleotide 1876 EcoNI site. To create full-length products, recombinant molecules were digested with BsrXI and XbaI, liberating an 1836-nucleotide product encoding the carboxyl half of the full-length EGF receptor, including the membrane spanning helix. These fragments were ligated to a 2100-nucleotide 

To make permanent MDCK cell lines, cells seeded at a density of 10^6 cells/100-mm tissue culture dish and incubated for 48 h post-transfection. Exogenously expressed human EGF receptors are readily detected in both cell types in CHO cells were transfected using DNA mixed with Lipofectin transfection reagent (GIBCO BRL, Gaithersburg, MD) (26, 33). To introduce a specific amino acid substitution (bold italics), a DraIII site (underlined) to facilitate subcloning, and a silent SacI site (double underlined) to facilitate recombination writing with a specific amino acid sequence: R662T, 5/CCTCGAGTTATATGAGCACTCCAGAAGC-3; S668P, 5/GGCTCTAGATGAGCACTCCAGAAGC-3; T670I, 5/GGCGTTAGATGAGCACTCCAGAAGC-3; and P670L, 5/GGCGTTATCTGGTGTCGCTCCAGAAGC-3. PCR products were digested at an EcoNI site located at EGF receptor nucleotide 1876 and the DraIII site located at the 5'-end of the PCR products, and ligated to full-length EGF receptor sequences cloned in pBK-CMVlacDIII digested with the same enzymes. When sequenced, the recombinant products had an internal deletion, due to a second previously unrecognized EcoNI site located 5' to the nucleotide 1876 EcoNI site. To create full-length products, recombinant molecules were digested with BsrXI and XbaI, liberating an 1836-nucleotide product encoding the carboxyl half of the full-length EGF receptor, including the membrane spanning helix. These fragments were ligated to a 2100-nucleotide 

PCR primers were designed using the DNASTAR software package (DNASTAR, Inc., Madison, WI). PCR amplifications were carried out using a RoboCycler 40 temperature cycler (Stratagene Cloning Systems, La Jolla, CA). All PCR products and ligated recombinant products were sequenced by automated DNA sequencing (Cleveland Genomics, Cleveland, OH). Sequences were verified by analysis of genomic DNA recovered from permanent clonal cell lines.

**Transient Transfections and Permanent Cell Lines**—CHO cells were seeded at a density of 2 × 10^6 cells/100-mm tissue culture dish and transfected using DNA mixed with FuGENE 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) 5 h later. Filter-grown MDCK cells were transfected using DNA mixed with LipoFectin transfection reagent (Invitrogen, Rockville, MD) added to both sides of the filter. Transfected cells were assayed after one day for cell membrane-domain-specific protein expression 40–48 h post-transfection. Exogenously expressed human EGF receptors are readily detected in both cell types in CHO cells, because they lack endogenous receptors (39), and in MDCK cells, because exogenously expressed human receptors can be distinguished from endogenous canine receptors with species-specific antibodies (33). To make permanent MDCK cell lines, cells seeded at a density of 7 × 10^6 cells/100-mm tissue culture dish and transfected using DNA mixed with FuGENE 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) 5 h later. Filter-grown MDCK cells were transfected using DNA mixed with LipoFectin transfection reagent (Invitrogen, Rockville, MD) added to both sides of the filter. Transfected cells were assayed after one day for cell membrane-domain-specific protein expression 40–48 h post-transfection. Exogenously expressed human EGF receptors are readily detected in both cell types in CHO cells, because they lack endogenous receptors (39), and in MDCK cells, because exogenously expressed human receptors can be distinguished from endogenous canine receptors with species-specific antibodies (33).
10^6 cells/100-mm tissue culture dish were transfected with DNA-Lipopectin complexes as described previously (33). Cells that had been grown in selection medium containing G418 (0.8 mg/ml Geneticin, Invitrogen) for 10–14 days were labeled with the monoclonal antibody EGF-R1, specific for an external human EGF receptor peptide epitope (40, 41), followed by FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), for enrichment by sterile sorting on a flow cytometer (Cytofluorograph IIIs, Ortho Instruments, Westwood, MA).

Metabolic Labeling and Immunoprecipitations—Cells were rinsed twice and then preincubated in methionine and cysteine-free medium for up to 1 h, before metabolic labeling with [35S]Express Protein Labeling Mix (1175 Ci/mmol, PerkinElmer Life Sciences, Wellesley, MA), diluted in the amino acid-deficient medium supplemented with 10% dialyzed FBS and 0.2% BSA. Filter-grown MDCK cells were labeled from the basolateral surface. Labeling medium was replaced with complete MEM supplemented with a 10-fold excess of non-radioactive methionine and cysteine (chase medium), and cells were incubated for additional periods of time. Cells were lysed with 1% (w/v) Triton X-100 thionine and cysteine (chase medium), and cells were incubated for complete MEM supplemented with a 10-fold excess of non-radioactive methionine and cysteine (chase medium), and cells were incubated for additional periods of time. Cells were lysed with 1% (w/v) Triton X-100 in 0.1 M Tris, pH 7.4, supplemented with 2 mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, and 1 mM leupeptin, for immunoprecipitation with antibodies recognizing external epitopes in the human EGF receptor (EGF-R1) or DAF (gift of Ed Medof, Case Western Reserve University) absorbed to protein A-Sepharose CL-4B beads (Sigma Chemical Co., St. Louis, MO). After extensive washing, immunoprecipitates were solubilized with Laemmli buffer for SDS-PAGE (42). Protein precipitation was achieved by a 5-min incubation with 0.05 M Tris, pH 7.4, at room temperature. Cells were lysed with 1% Nonidet P-40 exactly as described above, and equal aliquots of total cell protein were separated by SDS-PAGE. Precipitates were solubilized with Laemmli buffer for SDS-PAGE (42), denatured, and resolved by SDS-PAGE exactly as described in the previous section.

Membrane Domain-specific Surface Immunoprecipitation—Filter-grown MDCK cells that had been metabolically labeled were rinsed twice with ice-cold MEM supplemented with 25 mM HEPES, pH 7.4, and 1% BSA (M/H/B) and then incubated with EGF-R1 ascites (10 μl/ml) and the same side of the filter for 1 h on ice. Cells were rinsed four times with PBS supplemented with 1% BSA and then lysed with 1% (w/v) Nonidet P-40 in 0.1 M Tris, pH 6.8, supplemented with 15% (w/v) glycerol, 2 mM EDTA, 1 mM EGTA, and protease inhibitors. Cell lysates were added directly to protein A-Sepharose beads to capture surface-exposed EGF receptors, and immunoprecipitates were washed, solubilized, and resolved by SDS-PAGE exactly as described in the previous section.

Confocal Laser Scanning Microscopy—Filter-grown cells were rinsed three times with ice-cold MEM supplemented with 0.2% BSA and then incubated with ~10^14 125I-EGF for 2 h at 4 °C. Receptor-graft mouse EGF (Toyobo Biochemicals, Osaka, Japan) was labeled with 125I (carrier-free, >350 mCi/ml, PerkinElmer Life Sciences) using chloramine-T. Cells were rinsed three times with the MEM/BSA solution and then incubated with 2 mM disuccinimidyl suberate (sulfo-NHS-SS-biotin, Pierce Rockford, IL) in solution of 0.1 M HEPES, pH 7.4, supplemented with 120 mM NaCl, 50 mM KCl, 8 mM glucose, and 1.2 mM MgSO4 for 15 min at room temperature. The reaction was quenched by a 5-min incubation with 0.05 M Tris, pH 7.4, at room temperature. Cells were lysed with 1% Nonidet P-40 exactly as described above, and equal aliquots of total cell protein were separated by SDS-PAGE.

RESULTS

Residues Arg662, Pro667, and Pro670 Are Required for Accurate Basolateral Localization of Cytoplasmically Truncated c'-674 Molecules—The amino acid sequence of the originally identified a 22-amino acid juxtapemembrane domain necessary for basolateral localization of cytoplasmically truncated EGF receptors is shown in Fig. 1A (26, 33). We sought to characterize the molecular basis of basolateral localization, by focusing on several consensus amino acid protein interaction motifs located in this region. Examination of the sequence revealed the existence of a two consensus leucine-based motifs, located at Leu656-Leu659 and Leu666-Val669 (Fig. 1A). Although predominantly characterized in endocytic pathways (reviewed in Ref. 18), leucine-based motifs are implicated in basolateral sorting of at least two other membrane proteins (15, 16) (also see Table I). Because leucine-based signals are impaired by dialanine substitution (18), we changed each of the leucine-based signals to alanines, either individually (P675STOP-LL-2xA or P675STOP-LV-2xA) or in combination (P675STOP-LL-LV-4xA), in the molecular setting of a c'-674 receptor. In each instance, the cytoplasmically truncated mutant receptors were delivered to the basolateral surface with approximately the same efficiency as wild-type c'-674 receptors (Fig. 2). In addition, all of the mutant receptors exhibited normal basolateral localization based on CLSM analysis (see Fig. 3 for P675STOP-LV-2xA; others not shown).

This region also has a proline-rich segment, residues 662-PLTP870, whose sequence is characteristic of protein interaction motifs with a polyproline core PXE, where X denotes any amino acid (reviewed in Ref. 43). Recognition by cognate protein interaction partners is often dictated by the location of a positively charged residue relative to the PXE core. Thus, proline residues Pro667 (45) and Pro670 (46) and the positively charged residue Arg662, as well as two negatively charged glutamic residues Glu663 and Glu673, were altered by mutagenesis in cytoplasmically truncated c'-674 receptors (Fig. 1B). All of the residues were changed to alanines except for Arg662, which was changed to a threonine to mimic the residue found in the corresponding region of ErbB2 (44). The percentage of receptor mutants with individual substitutions at Arg662, Pro667, or Pro670 that were delivered to the apical surface ranged from 25 to 30%, which was significantly higher than the 10% apical delivery characteristic of wild-type c'-674 receptors (Fig. 2) or full-length molecules (26, 33). The same three mutations were also associated with higher than expected steady-state apical expression following domain-specific human receptor staining and CLSM (Fig. 3). These results suggest that a motif containing a polyproline core (967PPX870) and an amino-terminal positively charged residue (Arg662) has an important role in basolateral localization of cytoplasmically truncated receptors.

The EGF Receptor Juxtamembrane Domain Has Multiple Basolateral Determinants—Although individual mutations at residues Arg662, Pro667, or Pro670 each had a modest effect on the efficacy and fidelity of basolateral sorting, none of the mutations was sufficient to completely inactivate the putative signal. Although not all basolateral signals are inactivated by a single point mutation (57), one possible explanation for this result is that the juxtamembrane domain has additional basolateral sorting information that is normally silent relative to the putative proline-based signal. To test this hypothesis, the polyproline core was removed by changing residue Leu664 to a premature stop codon by site-directed mutagenesis (Fig. 1B).
Permanent MDCK cell lines expressing human receptors truncated to Glu663 (or c’-663 receptors made with the L664STOP construct) were then evaluated for domain-specific expression of the recombinant molecule. Its membrane domain expression was compared with that of c’/H11032-651 receptors (made with the K652STOP construct), which lack the juxtamembrane domain and sort selectively to the apical membrane, and c’/H11032-674 receptors (made with the P675STOP construct), which exhibit high efficiency localization to the basolateral membrane (26, 33).

Biosynthetic delivery was evaluated by domain-specific surface immunoprecipitation to capture newly made human receptors following a pulsed metabolic label, and plasma membrane steady-state expression by domain-specific human 125I-EGF chemical cross-linking or human EGF receptor staining and CLSM. Similar to full-length wild-type EGF receptors, relatively few c’-674 receptors are delivered to the apical membrane, compared with c’-651 receptors lacking juxtamembrane residues, where ~60% of the newly synthesized molecules were delivered to the apical surface during a 3-h interval (Fig. 4A).

By comparison, c’-663 receptors exhibited an intermediate phenotype in the delivery assay, with ~30% of newly synthesized molecules targeted to the apical surface (Fig. 4A). At steady-state, the difference between c’-651 and c’-674 receptors membrane domain distribution was even more pronounced, with the vast majority of c’-651 receptors located at the apical surface based on results obtained by 125I-EGF cross-linking (Fig. 4B) or by CLSM (Fig. 4C). Although c’-663 receptors also have a predominantly apical localization, basolateral expression was still evident in both of the steady-state assays (Fig. 4, B and C). These data suggest that residues Lys652 through Glu663 likely contain additional sorting information, because c’-663 receptors retain a limited capacity for basolateral expression.

**Fig. 1.** Amino acid sequences of wild-type and mutated cytoplastically truncated EGF receptor molecules. A, schematic showing EGF receptor domain structure (top) and the amino acid sequence of the juxtamembrane region depicted using the single-letter code (below). The areas boxed in gray indicate the location of basolateral sorting information previously mapped to residues 652–674 at the cytoplasmic face of the transmembrane (TM) domain (26). Amino acids converted to other residues or to premature stop codons by site-directed mutagenesis are indicated in white lettering. CRI and CRII, cysteine-rich domains I and II, respectively. B and C, amino acid sequences of EGF receptor constructs encoding mutant juxtamembrane domain sequences in the context of cytoplastically truncated molecules (B) or in the intact protein (C). Figures showing results obtained with the different mutants are indicated in the right-hand column.
Critical residues (insofar as they are known) are underlined. The number of residues separating signals from transmembrane (TM), or amino or carboxyl termini, are also indicated.

| Protein                  | Basolateral sorting domain | Colinear with internalization signal | References |
|--------------------------|-----------------------------|--------------------------------------|------------|
| EGF receptor             | TM**3**RELVEPLTPSGEAA**11**COOH | –                                    | This study |
| EGF receptor             | TM**3**KRTLRLIIQGERE**12**COOH | –                                    | This study |
| IgG FC receptor          | TM**3**KKRQVDPYPPDLLEAATFAEENTTYSSLKH**18**COOH | +                                    | (55)       |
| LAP**6**                | TM**3**RQAQPPGVRHYVADGEHA-COOH | +                                    | (13)       |
| LDL receptor             | TM**3**NPYYQKTTEDHII**4**COOH | +                                    | (13)       |
| LDL receptor             | TM**3**QDGYSPSRQVMSLEDVAA-COOH | +                                    | (13)       |
| lgp120                   | TM**3**KKSKHAYRTI-COOH       | +                                    | (56)       |
| MHC II Ii                | NH**14**PMLGRPGAPESKCS-TM    | Partial                              | (16)       |
| MHC II II                | NH**21**PMLGRPGAPESKCS-TM    | –                                    | (16)       |
| plg receptor             | TM**3**KRHRNVRDSIGSYRT-COOH  | –                                    | (57)       |
| Transferrin receptor     | NH**26**SYTRFSLARQYDGDNSHVEMKLA**31**TM | +                                    | (58)       |
| VSV G protein            | TM**-**RVIHLCKHTKRRQIYTDKMNRLGK-COOH | +                                    | (14)       |

**Abbreviations:** LAP, lysosomal acid phosphatase; MHC II Ii, major histocompatibility complex II invariant chain; lgp 120, lysosomal glycoprotein 120; plg, polymeric immunoglobulin; TM, transmembrane; VSV, vesicular stomatitis virus.

Residues Leu**658** and Leu**659** Are Critical Determinants When the Carboxyl Terminal Polypatine Core Is Deleted—To identify additional sorting motifs in the region between residues 652 and 663, a L658A,L659A dialanine substitution was engineered into a mutant receptor with a L664STOP lacking the proline-based carboxyl determinant (L664STOP-LL-2xA in Fig. 1B). Using CLSM to examine filter-grown cells that had been transiently transfected and then stained with an EGF receptor antibody to an external human-specific epitope added to both sides of the cell simultaneously, we observed that this mutation did cause an increase in the apical expression of receptor proteins compared with wild-type c**-**663 receptors (Fig. 5B). Because mutation of the leucine-based motif had no effect on polarized expression of c**-**674 receptor receptors with an intact 667PXXP**670** core (Figs. 2 and 3), these data suggest that the proline-based determinant is dominant over one or both of the leucine-based motifs in molecules with a P675STOP.

To verify that this region has two basolateral determinants, we created an additional set of receptor mutants with compound mutations in each of the putative sorting determinants in the molecular setting of a c**-**674 receptor (P675STOP-PxxP-2xA in Fig. 5C). A mutant receptor with both proline residues changed to alanines was also made and analyzed by CLSM (P675STOP-PxxP-2xA in Fig. 5C). A majority of truncated molecules was present on the apical membrane when either of the compound mutations was expressed in combination with a P675STOP. Apical expression of the P675STOP-PxxP-2xA receptor, however, appeared to be slightly greater than that of the P675STOP-PxxP-2xA. To obtain quantitative data, three of these constructs (LL-LV-4xA, PxxP-2xA, and LL-LV-PxxP-6xA) were used to make permanent cell lines with uniform mutant protein expression. As shown in Fig. 6A, all of the mutant receptors exhibited elevated apical expression compared with cytoplasmically truncated wild-type c**-**674 receptors, where 5% of the total receptors located at the plasma membrane are localized to the apical surface, as judged by **125**I-EGF cross-linking. Approximately 20% of the receptors with both leucine-based motifs changed to alanines, and 33% of...
the receptors with a PxxP-2x substitution, were present on the apical surface. Apical expression of receptors with all three signals converted to alanines was 65%, consistent with the results obtained by transient transfection. Permanent cell lines expressing the mutant receptors were also analyzed by domain-specific human EGF receptor staining followed by CLSM (Fig. 6B), to show that the entire cell population had increased apical expression. These results support the hypothesis that the failure to completely reverse polar sorting of c'-674 receptors by introducing mutations in the polyproline-based signal is due to compensatory activity of a functionally redundant signal that is normally silent.

The Same Determinants Identified in Truncated EGF Receptors Also Regulate Basolateral Localization of DAF-EGF Receptor Chimeras—We have shown previously that residues EGF receptor residues Lys652 to Ala674 constitute a dominant autonomous basolateral localization determinant in reporter molecules containing the luminal domain from decay accelerating factor (DAF) attached to transmembrane and cytosolic sequences from the EGF receptor. To test whether any of the amino acid substitutions associated with increased apical expression of cytoplasmically truncated EGF receptors had a similar effect in another molecular setting, two of the mutations, L664STOP and P675STOP, were expressed in the...
protein chimeras (DAF-663 and DAF-674A, respectively, in Fig. 7A). Like other GPI-anchored membrane proteins, native DAF partitions into Triton-insoluble membrane rafts during polarized sorting to the apical surface of polarized cells (45). DAF is partially soluble in Triton, if cell extracts are incubated at 37°C instead of 4°C (Fig. 7B, left and middle panels) (46). Replacement of the GPI anchor with sequences encoding the transmembrane domain and seven amino acids from the EGF receptor cytosolic tail sequence is sufficient to render the protein chimera partially apical localization, compared with wild-type DAF, which was detected predominantly on the basolateral surface. Protein chimeras with either a L664STOP (DAF-663) or a P675STOP with a P667A substitution (DAF-674A), however, were detected on both sides of the cell, similar to results obtained when the same mutations, are expressed in the molecule Arg662, Pro667, and Pro 670 are each necessary for accurate basolateral localization of intact EGF receptors. Pro667 and Glu673 were individually mutated in cDNAs encoding full-length receptors (see Fig. 1C). To design a PCR mutagenic primer with favorable secondary structure, it was necessary to convert Pro667 to a leucine residue instead of an alanine. Pro667 and Glu673 were
with individual amino acid substitutions at Arg662, Pro667, or B steady state by CLSM. As shown in Fig. 8, mutant receptors membrane domain-specific human EGF receptor expression at were then transfected with each of these constructs to assess levels of plasma membrane-exposed EGF receptor proteins, and EGF receptor expression was analyzed by domain-specific surface immunoprecipitation of newly synthesized molecules (Fig. 9). The data in Fig. 9 indicates that receptors with a P667A substitution exhibit substantially elevated delivery to the apical membrane, compared with wild-type receptors and mutant receptors with an E673A substitution, where the vast majority is delivered to basolaterally.

**FIG. 8.** Transient expression of full-length EGF receptors containing single amino acid substitutions. A, cells that had been transfected with cDNAs encoding wild-type human EGF receptor (EGFRwt) or intact receptors containing the amino acid substitutions listed in the figure, were metabolically labeled 48 h later. Cell lysates were immunoprecipitated with a human EGF receptor-specific antibody, and immunoprecipitates were resolved by SDS-PAGE. Molecular weight standards: myosin, 200,000; α-galactosidase, 116,200; phosphorlyase B, 97,400; bovine serum albumin, 66,200. B, filter-grown cells were fixed and stained with EGF-R1 added to either the apical or the basolateral surface, followed by a FITC-conjugated secondary antibody.

**DISCUSSION**

We have shown that two different types of signals located in the juxtamembrane region regulate basolateral EGF receptor expression in polarized epithelial cells. One class of signals conforms to leucine-based signals already characterized in other molecules, based on its dependence on residues 659LL660. The second is dependent on two proline residues, Pro667 and Pro707, with a possible contribution by Arg662. The presence of functionally redundant signals is the most likely explanation for the fact that basolateral sorting is not completely reversed unless critical residues are mutated in both of the signals. In contrast, alternative signals may need to be used to regulate EGF receptor transport by distinct mechanisms. Furthermore, alternative signals may regulate polarized sorting from different membrane compartments (e.g. TGN versus endosomes) (6) or in other cell types where asymmetrically distributed proteins are sorted differently than in MDCK cells (47). Given the complexity of polarized membrane protein sorting, it is also possible that distinct sorting motifs mediate different transport steps along a common secretory pathway. In that event, the dominant phenotype of receptors with a defective PXXP707 signal suggests that it acts downstream of 658LL659.

Although our data suggest that residues comprising the proline-dependent motif are necessary for restricting native EGF receptor expression to the basolateral membrane in polarized cells, additional signals not yet identified may also contribute to the final membrane phenotype. The additional signals may direct independent sorting events in the secretory pathway or specify post-TGN events such as vesicle docking or membrane retention (48). In fact, essential basolateral localization signals
Basal Cell EGF Receptor Expression

have been identified in the Caenorhabditis elegans EGF receptor homologue (49), as well as the mammalian ErbB2 receptor (50), that bind to proteins with PDZ domains whose expression is critical for accurate receptor localization in polarized cells. Although the human EGF receptor lacks a consensus PDZ-binding motif, other intrinsic signals could fulfill a similar role at the basolateral membrane. For example, an actin-binding domain mapped to EGF receptor carboxyl-terminal residues 984–996 several years ago (51) could contribute to basolateral membrane retention, by tethering receptors to the actin cytoskeleton. This is consistent with our previous studies showing that expression of cytoplastically truncated receptors lacking an intact actin-binding motif is not restricted to the basolateral domain, despite their highly efficient basolateral delivery in the secretory pathway (26). However, because the EGF receptor actin-binding motif is located in a domain that also contains at least two known internalization signals (34), understanding the molecular basis of any signals in this region that might be involved in polarized expression is not so clear-cut. Clarifying the role of multiple sorting signals all contributing to basolateral localization will help illuminate the complex network of protein-protein interactions that organize and coordinate receptor trafficking and signaling. It may also provide a rationale for understanding how EGF receptor sorting is disrupted in different forms of inherited polycystic kidney disease, if individual disease gene products target distinct protein-protein interactions acting sequentially in a common sorting pathway.

Although many different types of protein-protein interactions are mediated by proline-rich motifs, this represents the first basalolateral localization determinant identified with these molecular characteristics (Table I). Only one other signal, residues RRPPGAEKS at major histocompatibility complex II invariant chain (Table I), has a similarly proline-rich core (underlined) (16). The best-studied proline-based interactions are those involving protein partners with SH3 or WW domains identified in numerous signal transduction and cytoskeletal protein adaptors (reviewed in Ref. 43). In addition to critical proline core residues, these interactions are also dependent on other residues in the core itself as well as in the nearby vicinity. SH3 ligands, for example, can be classified as class I or class II depending on the location of a positively charged residue relative to the proline core. Although the EGF receptor basolateral sorting signal could be classified as a class I SH3 ligand, based on its dependence on a positively charged residue (Arg<sup>662</sup>) on the amino-terminal side of a proline core (667P<sup>XX</sup>670), the diversity of protein interactions mediated by proline-rich sequences necessitates further investigation. The proline cores of many SH3 ligands also have critical serine or threonine residues, whose phosphorylation regulates binding affinity (43). Interestingly, the proline core of the dominant EGF receptor basolateral sorting signal contains the major EGF receptor mitogen-activated protein kinase substrate Thr<sup>669</sup> (52, 53), raising the possibility that sorting signal recognition is regulated by phosphorylation.

Several key questions remain. First, what is the nature of the protein interactions mediated by these basolateral localization signals? AP-1-regulated sequestration of membrane cargo with tyrosine-dependent sorting signals into clathrin-coated transport vesicles is thus far the only mechanism implicated in regulated sorting of basolateral proteins (23, 24). However, the nature of the EGF receptor sorting signals, as well as the accuracy of EGF receptor sorting in LLC-PK<sub>1</sub> cells lacking the AP-1 isoform necessary for polarized sorting (37), suggests that these motifs underlie a novel mechanism. Whether EGF recep-
Basolateral EGF Receptor Expression

43. Kay, B. K., Williamson, M. P., and Sudol, M. (2000) *FASEB J.* 14, 231–241
44. DiFiore, P. P., Helin, K., Kraus, M. H., Pierce, J. H., Artrip, J., Segatto, O., and Bottaro, D. P. (1992) *EMBO J.* 11, 3927–3933
45. Lisanti, M., LeBivic, A., Saltiel, A., and Rodriguez-Boulan, E. (1990) *J. Membr. Biol.* 113, 155–167
46. Brown, D. A., and Rose, J. K. (1992) *Cell* 68, 533–544
47. Keller, P., and Simons, K. (1997) *J. Cell Sci.* 110, 3001–3009
48. Yeaman, C., Grinstaff, K. K., and Nelson, W. J. (1999) *Physiol. Rev.* 79, 73–98
49. Whitfield, C. W., Benard, C., Barnes, T., Hekimi, S., and Kim, S. K. (1999) *Mol. Biol. Cell* 10, 2087–2100
50. Borg, J. P., Marchetto, S., Bivic, A. L., Ollendorff, V., Jaulin-Bastard, F., Saito, H., Fournier, E., Adelaide, J., Margolis, B., and Birnbaum, D. (2000) *Nat. Cell Biol.* 2, 407–414
51. den Hartigh, J. C., Henegouwen, P. M. P. v. B. e., Verkleij, A. J., and Boonstra, J. (1992) *J. Cell Biol.* 119, 349–355
52. Countaway, J. L., Northwood, I. C., and Davis, R. J. (1989) *J. Biol. Chem.* 264, 10828–10835
53. Takashima, K., Griswold-Prenner, I., Ingbreitsen, T., and Rosner, M. R. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 2520–2524
54. Kil, S., and Carlin, C. (2000) *J. Cell. Physiol.* 185, 47–60
55. Prill, V., Lehmann, L., Figura, K. v., and Peters, C. (1993) *EMBO J.* 12, 2181–2193
56. Hunziker, W., Harter, C., Matter, K., and Mellman, I. (1991) *Cell* 66, 907–920
57. Casanova, J. E., Apodaca, G., and Mostov, K. E. (1991) *Cell* 66, 65–75
58. Dargemont, C., LeBivic, A., Rothenberger, S., Iacopetta, B., and Kuehn, L. C. (1993) *EMBO J.* 12, 1713–1721
