Deletions in the Cytoplasmic Domain of the Polymeric Immunoglobulin Receptor Differentially Affect Endocytotic Rate and Postendocytotic Traffic*

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We have examined the function of the cytoplasmic domain of the polymeric immunoglobulin receptor (pig-R) by producing two separate deletions in the cytoplasmic domain of the pig-R, expressing the mutant receptors in polarized MDCK cells, and analyzing each for their effects on receptor and ligand traffic. Deletion of the C-terminal 30 amino acids (726-756) reduces the rate of internalization of receptor-bound ligand from the basolateral surface. However, this mutation has no effect on delivery of receptor from the Golgi to the basolateral surface or the post-endocytotic traffic of receptor and ligand. Mutation of a tyrosine at position 734 to serine produces a receptor with a similar phenotype. If residues 670-707 are deleted from the middle of the cytoplasmic domain, both basolateral delivery and internalization are unaffected. However, unlike wild type, after endocytosis from the basolateral surface, both receptor and ligand are largely degraded. We reported previously that deletion of the entire cytoplasmic domain prevents the basolateral delivery of newly synthesized receptor (Mostov, K. E., de Bruyn Kops, A., and Deitcher, D. L. (1986) Cell 47, 359-364). In contrast, the mutants reported here are delivered to the basolateral surface, suggesting that only residues 653-669 and/or 708-725 are necessary for basolateral delivery. Thus, different deletions in the cytoplasmic domain of the pig-R can produce mutant receptors which alter different aspects of receptor traffic.

The polymeric immunoglobulin receptor (pig-R) is responsible for the receptor-mediated transcytosis of dimeric IgA and pentameric IgM (polymeric immunoglobulins, pig) across various epithelia into external secretions. The itinerary of the pig-R begins with synthesis in the rough endoplasmic reticulum and processing in Golgi stacks, followed by delivery to the basolateral surface of polarized epithelial cells. At the basolateral surface, receptor binds pig, and the receptor-ligand complex is internalized via coated pits (Geuze et al., 1984). Receptor-ligand complexes pass through the endosomal compartment and are then delivered to the apical surface (Takahasi et al., 1982; Geuze et al., 1984). At or near the apical surface of the cell, receptor is cleaved to secretory component (SC), releasing pig with SC.

Because of this complex sorting pathway, the receptor mediated transcytosis of pig serves as an excellent model system for the study of receptor and ligand traffic in polarized epithelial cells. In order to examine the factors that control this traffic, we have expressed the cDNA for rabbit pig-R in Madin-Darby canine kidney (MDCK) cells by utilizing a retroviral expression vector (Mostov and Deitcher, 1986). MDCK cells are cultured on suspended permeable filters such that the basolateral and apical domains are biochemically separate. Using this system, we have shown that the biosynthetic and transcytotic pathways of the pig-R mimic that found in vivo (Mostov and Deitcher, 1986).

We have suggested that the cytoplasmic domain of the pig-R contains structural determinants or signals that play a role in receptor and ligand traffic since deletion of the entire cytoplasmic domain of the pig-R (tail-minus mutant) prevents both basolateral delivery of newly synthesized receptor and internalization (Mostov et al., 1986). A role for the cytoplasmic domain in the intracellular traffic of several other integral membrane proteins has been proposed. For example, Gonzalez and co-workers (1987) have suggested an important role for the cytoplasmic domain of the vesicular stomatitis virus G-protein in the delivery of the newly synthesized protein to the basolateral surface of epithelial cells. The cytoplasmic domains for the low density lipoprotein (LDL) receptor (Lehrman et al., 1985), transferrin receptor (Rothengerber et al., 1987), and epidermal growth factor receptor (Schlessinger, 1986) have been demonstrated to be important for internalization. Interestingly, point mutations of the tyrosine residue(s) within the cytoplasmic domains of the LDL receptor (Davis et al., 1987), the transferrin receptor (Jing et al., 1990), and mannose 6-phosphate receptor (Lobel et al., 1989) impair rapid internalization of their respective ligands. The cytoplasmic domain may contain several restricted segments which control different aspects of receptor traffic. For example, Lobel and co-workers (1989) have recently shown that rapid endocytosis and efficient ligand sorting are affected by different mutations in the cytoplasmic domain of the 275-kDa mannose 6-phosphate receptor.

We have now extended the analysis of the function of the cytoplasmic domain of receptor molecules in receptor and...
ligand traffic. We have constructed two cytoplasmic domain deletion mutants of the plg-R, expressed them in polarized MDCK cells, and have analyzed each for their effects on receptor and ligand traffic in a polarized epithelial cell line. Our results indicate that basolateral delivery of newly synthesized plg-R, rapid internalization of receptor-bound ligand, and delivery of ligand after endocytosis are affected by different mutations of the cytoplasmic domain of the receptor.

**EXPERIMENTAL PROCEDURES**

**Growth of MDCK Cells on Suspended Filters—** MDCK cells were cultured on 0.4-μm-pore Transwell chambers (Costar Corp., Cambridge, MA). These units were placed in a 24-well tissue culture plate (Corning) and pre-wet with medium for 5 min at room temperature. Cells from a confluent 10-cm tissue culture dish were removed by trypsinization, with washed, with medium, and resuspended in 10 ml of medium. An aliquot of each cell suspension (0.2 ml) was added to each chamber. Fresh medium was added daily. Chambers were used for study after 3 or 4 days of growth. Cells were grown in Eagle’s minimal essential medium (MEM) with 10% fetal calf serum, penicillin, and streptomycin. MDCK cells which express the wild-type pig-R, but in varying angles. Three clones expressing the highest levels of each mutant plg-R were selected for further study. All three clones for each mutation had similar phenotypes by pulse-chase and transcytosis analysis and formed intact polarized monolayers. One MDCK cell clone for each mutation was arbitrarily selected for this report.

**Resistance Measurements for MDCK Cells—** MDCK cells cultured on suspended filters were used to verify the polarity of the various cell lines used. First, we measured the transepithelial resistance of filter-grown cells with a Millicell-ERS resistance measuring device and electrodes (Millipore Corp.). Measurements were carried out on 4-day-old cells, in quadruplicate, at room temperature and were corrected for a blank filter. Resistance measurements were performed in both the apical and basolaterally delivered molecules, and the amount of native plg-R mutant receptor did not vary significantly (range: 65–88 ohms/cm²).

**Second, we examined the secretion of an endogenous group of 35-45-kDa peptides which are synthesized in MDCK cells as an 80-kDa precursor (Breitfeld et al., 1989a) was utilized for the construction of the plg-R mutants.**

**To generate the 725-pig-R, the above plg-R construction in M13 was digested with AvrII at 2441, followed by digestion with Bal-31 exonuclease ("slow" form of the enzyme, BD, New Haven, CT) for 1–5 min. At various time points, aliquots were hybridized with a pig-R specific probe (Breitfeld et al., 1989; Mostov and Deitcher, 1986; Breitfeld et al., 1989a) or the two plg-R mutations described in Fig. 1 were used for study.**

**Construction of Mutant plg-R Clones—** All recombinant DNA manipulations utilized standard techniques (Maniatis et al., 1982). The replicative form of the M13 Mp8 cloning vector containing the coding region for pig-R was isolated from M13 with the restriction enzyme BglII sites at the ends of the coding region (Mostov and Deitcher, 1986; Breitfeld et al., 1989a) was utilized for the construction of the plg-R mutants.

**For the Δ6670-707 plg-R, we isolated a fragment of the pig-R containing a single Smal restriction site at nucleotide 2261 by digesting the plg-R expression in M13 Plb. The 1.2-kilobase pair fragment (from nucleotide 1261 of the pig-R coding region to the Pst site of the M13mp8 polylinker, which is 3' to the coding region) was isolated by agarose electrophoresis. This fragment was ligated into the Pst site of the M13mp8 polylinker. This construction was digested with Smal and then with the exonuclease Bal 31 for 2, 6, or 20 min.**

**The second level of synthetic medium containing 0.6% BSA was pre-warmed 24-well tissue culture plates with 200 and 400 μl of MEM with 10% fetal calf serum in the apical and basolateral chambers, respectively. The incubation was continued at 37 °C for the designated chase period. At the end of the chase period, the filters were transferred to pre-warmed 24-well tissue culture plates and 200 μl of medium containing 0.6% BSA, 20 mM Hepes (pH 7.3), and 25 μg/ml trypsin in the basolateral medium and soybean trypsin inhibitor at 200 μg/ml in the apical medium. To detect apically delivered molecules, the chase was performed with MEM containing 0.6% BSA, 20 mM Hepes (pH 7.3), and 25 μg/ml trypsin in the basolateral medium and soybean trypsin inhibitor at 200 μg/ml in the apical medium. To detect apically delivered molecules, the chase was performed with 25 μg/ml trypsin in the apical medium.**

**To detect basolaterally delivered molecules, the chase was performed with MEM containing 0.6% BSA, 20 mM Hepes (pH 7.3), and 25 μg/ml trypsin in the basolateral medium and soybean trypsin inhibitor at 200 μg/ml in the apical medium. To detect apically delivered molecules, the chase was performed with 25 μg/ml trypsin in the apical medium.**

**At 37 °C for the designated chase period. At the end of the chase period, the filters were transferred to pre-warmed 24-well tissue culture plates with 200 and 400 μl of MEM with 10% fetal calf serum in the apical and basolateral chambers, respectively. The incubation was continued at 37 °C for the designated chase period. At the end of the chase period, the filters were transferred to pre-warmed 24-well tissue culture plates and 200 μl of medium containing 0.6% BSA, 20 mM Hepes (pH 7.3), and 25 μg/ml trypsin in the basolateral medium and soybean trypsin inhibitor at 200 μg/ml in the apical medium. To detect apically delivered molecules, the chase was performed with 25 μg/ml trypsin in the apical medium.**

**Deduction of Metabolically Labeled plg-R at the Cell Surface—** MDCK cells which express the wild-type or mutant plg-R were metabolically labeled with [35S]cysteine for 10 min at 37 °C as described above. After the pulse period, cells were chased for various times in synthetic medium at 37 °C. At the end of the pulse period, the filters were transferred to pre-warmed 24-well tissue culture plates with 200 and 400 μl of MEM with 10% fetal calf serum in the apical and basolateral chambers, respectively. The incubation was continued at 37 °C for the designated chase period. At the end of the chase period, cells as well as the apical and basolateral media were harvested and immunoprecipitated with guinea pig anti-rabbit SC antibody, as described previously (Breitfeld et al., 1989a). Immunoprecipitates were analyzed by SDS-PAGE and fluorography.

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**Localization of Anti-SC Fab Fragments and dIa—** The generation of affinity purified guinea pig anti-rabbit SC Fab fragments has been described previously (Breitfeld et al., 1989a). The anti-SC Fab fragments and human dIa (gift of Dr. M. Schiff, University of Toronto) were iodinated by the iodine monochloride method (Goldstein et al., 1983) to a specific activity of 5 × 10⁶ cpm/μl. Unincorporated 125I was removed by dialysis.

**Time Course of Internalization—** For these experiments, cells were manipulated in medium consisting of MEM plus 0.6% BSA and 20 mM Hepes (pH 7.3). MDCK cells, cultured on suspended filters, were rapidly cooled to 4 °C and were allowed to bind 125I-Anti-SC Fab fragments (at 4 μg/ml) for 2 h. To remove nonspecifically bound molecules, cells were washed extensively with medium at 4 °C for 60 min. To determine the rate of internalization, cells were rapidly warmed to 37 °C for the designated internalization period (from 1 to 12 min) and then rapidly cooled to 4 °C. At the end of the internalization period, the basolateral medium was harvested, and the amount of radioactivity was quantitated in a Packard γ counter (Packard Instrument Co., Downers Grove, IL). To determine the amount of ligand remaining at the cell surface, the basolateral surface of cells was incubated with a mixture of 50 μg/ml chymotrypsin and 50 μg/ml proteinase K in PBS in two successive 30-min incubations at 4 °C. Cells were then washed for 15 min at 4 °C with medium. The amount
of radioactivity was determined in the protease-sensitive fraction (cell surface) and the protease-resistant fraction (intracellular), as above.

Post-endocytotic Delivery of Ligand—For experiments that examine the delivery of endocytosed anti-SC Fab fragments, filter-grown MDCK cells expressing wild-type or mutant receptors were allowed to internalize 125I-anti-SC Fab fragments (40 pg/ml) at 37 °C for 10 min from the basolateral surface. Cells were then washed four times with medium and placed into culture wells containing fresh MEM, 0.6% BSA, 20 mM Hepes (pH 7.3), all at 37 °C. Medium from the apical or the basolateral chamber was harvested at the appropriate time and made to 15% trichloroacetic acid. Samples precipitated for 1 h at 4 °C, and the soluble (degraded) and insoluble (intact) material was separated by centrifugation at 15,000 × g for 15 min at 4 °C.

Filters were counted directly at the end of the experiment. MDCK cells that do not express the plg-R were analyzed in parallel to control for nonspecific binding and fluid phase uptake of ligand. Values obtained at each time point for these cells were subtracted as nonspecific background. Radioactivity was determined as described above.

Ligand Dissociation—All manipulations were carried out either in MEM, 0.6% BSA, 20 mM Hepes (pH 7.3) with 0.2 M sucrose or in PBS containing 0.6% BSA and adjusted to pH 5.3 by adding 10 mM acetic acid. After preincubation of cells in the appropriate medium, ligand (either 125I-Fab at 4 μg/ml or 125I-dlgA at 2 μg/ml) was bound to the basolateral surface of filter-grown cells. Incubation was for 2 h at 4 °C followed by five washes over a 20-min period at 4 °C to remove nonspecifically bound ligand. Filters were then transferred to the appropriate media at 37 °C. The basolateral medium was sampled and replaced after 1.25, 2.5, 5, 10, and 20 min. Ligand released into the basolateral medium was determined by γ counting. All points were corrected for nonspecific binding of ligand by subtracting the results from parallel filters containing MDCK cells which do not express any form of the plg-R.

RESULTS

The wild-type plg-R is a 755-amino acid polypeptide chain which contains one putative membrane spanning domain of 23 amino acids and a C-terminal cytoplasmic domain of 103 amino acids, residues 653–755 (Mostov et al., 1984). We have shown previously that when 101 C-terminal amino acids of the cytoplasmic domain are deleted (tail-minus mutant), the mutant receptor expressed in MDCK cells not only fails to internalize ligand but is delivered after biosynthesis to the apical surface rather than the basolateral surface (Mostov et al., 1986). We have now further investigated the function of the cytoplasmic domain of the plg-R by constructing two deletion mutations within the cytoplasmic domain of the plg-R. We describe their expression and functional analysis in this report. These mutations are summarized in Fig. 1.

Expression and Biosynthesis of plg-R Mutants in MDCK Cells—Using a retroviral vector and the ψ packaging system as described previously (Mostov and Deitcher, 1986; Breitfeld et al., 1989a), both the wild-type and mutant plg-Rs were expressed in MDCK cells. To examine the biosynthesis of the mutant plg-Rs, MDCK cells expressing the wild-type plg-R or a mutant plg-R were pulse-labeled with [35S]cysteine for 20 min at 37 °C and chased for various periods of time. Cells, as well as apical and basolateral media, were harvested, immunoprecipitated with anti-SC antibody, and analyzed by SDS-PAGE and fluorography. Fig. 2A displays the pulse-chase analysis of the 725t plg-R. The results are similar to those found with wild-type receptor except that the 725t plg-R migrates faster on SDS-PAGE, since 30 amino acids have been deleted from the cytoplasmic domain. In particular, like wild-type receptor, after the mature product is formed, SC is released into the apical medium (Fig. 2B, lanes 8, 10, and 12).

Fig. 2B displays the pulse-chase analysis of the 725t plg-R. The results are similar to those found with wild-type receptor except that the 725t plg-R migrates faster on SDS-PAGE, since 30 amino acids have been deleted from the cytoplasmic domain. In particular, like wild-type receptor, after the mature product is formed, SC is released into the apical medium (Fig. 2B, lanes 8, 10, and 12).

Fig. 2C demonstrates that the Δ670–707 plg-R is synthe-
sized as a precursor (lane 1) which is converted to a doublet of 88 and 90 kDa by 1 h of chase (lane 2), reflecting processing of its oligosaccharide side chains to the complex type in the Golgi. Much like wild-type receptor, this newly synthesized Δ670–707 pig-R is degraded during the subsequent chase period (lanes 3–5), but, unlike wild-type, no SC is released into the apical medium (lanes 6, 8, 10, and 12). Rather, the Δ670–707 pig-R is apparently degraded to products that are too small to be detected by our immunoprecipitation/SDS-PAGE protocol.

To further characterize the degradation of the Δ670–707 pig-R, we asked if the presence of ligand would have an effect on this degradation. Ligand binding can alter the fate of several other receptors resulting in receptor degradation in acidic compartments such as late endosomes and lysosomes (Stoscheck and Carpenter, 1984). Therefore, cells expressing the Δ670–707 pig-R were metabolically labeled and chased for various times, either in the absence (Fig. 3A) or presence of ligand (dIgA at 10 μg/ml, Fig. 3B). The cells and media were harvested and immunoprecipitated with anti-SC antibody. We determined the rate of Δ670–707 pig-R degradation by laser densitometry of the immunoprecipitated bands. Fig. 3A demonstrates that the Δ670–707 pig-R is rapidly degraded in MDCK cells with a half-life of approximately 1 h. At 4 h of chase, less than 10% of the Δ670–707 pig-R remains in cells (Fig. 3A, lane 4), yet there is no SC released into the medium (lanes 5 and 6). Fig. 3B demonstrates that added ligand has very little effect on the rate of Δ670–707 pig-R degradation (compare with Fig. 3A). As in control Δ670–707 pig-R cells, no SC is released into the medium after 4 h of chase (lanes 5 and 6, compare Fig. 3, A and B). This suggests that the degradation of newly synthesized Δ670–707 pig-R is independent of ligand.

Biosynthetic Delivery of Wild-type and Mutant pig-Rs—To determine whether newly synthesized receptor is delivered to either the apical or basolateral surface of MDCK cells, a trypsin assay was developed. This assay was modeled after that utilized by Matlin and Simons (1984) to examine surface delivery of influenza virus hemagglutinin in MDCK cells. Our assay conditions were optimized with MDCK cells which express the wild-type pig-R, since we had shown previously, by an antibody-dependent assay, that over 90% of newly synthesized wild-type pig-R is delivered first to the basolateral surface (Mostov and Deitcher, 1986). We could not use this earlier assay for the Δ670–707 pig-R in particular since the antibody-dependent assay requires cleavage of the receptor to SC at the apical surface. Therefore, MDCK cells expressing either wild-type or mutant pig-R were pulse-labeled with [35S]cysteine for 10 min and chased for 45 min in the absence or presence of 25 μg/ml trypsin in the basolateral or apical medium. Soybean trypsin inhibitor at 200 μg/ml was included in the medium opposite to trypsin. The entire pulse and chase periods were performed at 37 °C. Newly synthesized receptor molecules which reach the surface exposed to trypsin will be digested, resulting in a decrease compared to control in the amount of immunoprecipitable protein.

Fig. 4A demonstrates the results for such an experiment with the wild-type pig-R. After the pulse period, the pig-R precursor is identified (lane 1). After the chase period of 45 min, the mature form of the receptor is identified (lane 2). If trypsin is included in the basolateral medium during the chase period, all newly synthesized receptor is digested (lane 3). Yet, if trypsin is included in the apical medium during the chase, little or no receptor is digested (lane 4). These results are
entirely consistent with those found previously (Mostov and Deitcher, 1986) and confirms that newly synthesized wild-type plg-R is delivered to the basolateral surface. In addition, the incubation with apical trypsin (lane 4) suggests that, under the conditions of our assay, apical trypsin is not entering the cellular compartments responsible for the delivery of newly synthesized receptor to the basolateral surface and not leaking across the monolayer to the basolateral surface. Moreover, we found that the tail-minus mutant, which lacks 101 residues of the cytoplasmic domain and is not basolaterally targeted, is not digested by adding trypsin to the basolateral medium (Casanova et al., 1990).

An identical experiment was performed with MDCK cells which express either the 725t plg-R (Fig. 4B) or the Δ670-707 plg-R (Fig. 4C). As with wild-type receptor, each of these plg-R mutants is delivered to the basolateral surface. Since exposure of cells to basolateral trypsin during the chase period digests newly synthesized mutant plg-Rs (lane 3, B and C). None reaches the apical surface during the 45 min of chase, since the addition of apical trypsin has no effect (lane 4, B and C). This data demonstrates that the wild-type plg-R as well as the mutant plg-Rs are vectorally delivered to the basolateral surface of MDCK cells after biosynthesis.

In a similar fashion, we examined the rate of delivery of newly synthesized wild-type and mutant receptor to the basolateral surface. After metabolic labeling, cells were chased for various periods of time without or with trypsin in the basolateral compartment. After immunoprecipitation and SDS-PAGE, the amount of receptor appearing at the basolateral surface was quantitated by laser densitometry. Fig. 5 demonstrates that 90% of wild-type plg-R reaches the basolateral surface by 45 min and the half-time for this process is approximately 25–30 min. The 725t plg-R appears at the basolateral surface with a slightly shorter half-time (no longer than 15 min) compared with wild-type, whereas the Δ670-707 plg-R follows roughly the same time course as wild-type receptor. Thus, we conclude that amino acid segments 670-707 and 726-755 are not required for delivery of newly synthesized receptor to the basolateral surface, although deletion of residues 726-755 may enhance the rate of transport to the surface. Of particular note, almost all newly synthesized Δ670-707 plg-R reaches the basolateral surface within the 45-min chase period and, thus, prior to its intracellular degradation.

Since we have shown previously that deletion of 101 amino acids of the 103 amino acid cytoplasmic domain of the plg-R prevents proper delivery of newly synthesized receptor to the basolateral surface (Mostov et al., 1986), we suggest that amino acids 663-669 and/or 708-725 are needed for this event.

Time Course of Internalization of Receptor-bound Ligand—To quantitate internalization of receptor-bound ligand from the basolateral surface, MDCK cells which express wild-type or mutant plg-R were allowed to bind radioiodinated Fab fragments derived from a polyclonal antibody raised against rabbit SC (anti-SC Fab fragments) at the basolateral surface for 2 h at 4 °C. We have shown previously that anti-SC Fab fragments function as native ligand (difA) (Breitfeld et al., 1989b). This substitution was required since radioiodinated difA, when utilized for basolateral binding assays, produces an unacceptable level of background binding at 4 °C as a consequence of nonspecific binding to the filter on which cells are cultured (Breitfeld et al., 1989b).

After extensive washing, cells were rapidly warmed to 37 °C for various periods of time up to 12 min. After the designated warm-up period, cells were rapidly cooled to 4 °C and exposed to proteolysis at the basolateral surface with both chymotrypsin and proteinase K at 50 μg/ml for 60 min at 4 °C. This proteolysis step efficiently strips the basolateral surface of cell surface bound noninternalized anti-SC Fab fragments, yet does not disrupt the MDCK cell monolayer (Breitfeld et al., 1989b). The amount of radioactivity was then determined in the basolateral warm-up medium, the protease-sensitive fraction (surface), and protease-resistant fraction (intracellular).

The percentage of ligand internalized as a function of time for wild-type and mutant plg-R is displayed in Fig. 6. For wild-type receptor, approximately 80% of bound anti-SC Fab fragments are internalized from the basolateral surface. This process is rapid, having a half-time of less than 1 min and reaching a maximum by 2.5 min. At 12 min, there is a decrease in the amount of intracellular anti-SC Fab fragments. This decrease is accompanied by an increase in the amount of surface-bound Fab fragments (Fig. 6).

% internalized

Fig. 5. Time course of delivery of newly synthesized wild-type and mutant plg-R to the basolateral surface. Cells expressing wild-type (closed squares), 725t (open squares), or Δ670-707 plg-R (open triangles) were cultured on suspended filters, metabolically labeled with [35S]cysteine for 10 min, and chased for various periods of time without or with trypsin in the basolateral compartment. After immunoprecipitation and SDS-PAGE, the amount of receptor appearing at the basolateral surface was quantitated by laser densitometry. The percentage of ligand internalized as a function of time was as follows: wild-type, 3848 cpm; 725t, 1089 cpm; Ser-734, 1169 cpm; tail-minus, 1177 cpm.
represents return of a fraction of basolaterally internalized ligand to the basolateral medium, as shown previously (Breitfeld et al.
1989b).

The \( \Delta 670-707 \) plg-R also rapidly internalizes anti-SC Fab fragments, reaching a maximum at 5 min with a half-time of less than 1 min. Thus, the time course of internalization for the \( \Delta 670-707 \) plg-R is similar to that of wild-type receptor, although the \( \Delta 670-707 \) plg-R is slightly slower. This suggests that the 38-amino acid deletion from 670 to 707 is not critical for the rapid internalization of receptor-bound ligand.

In contrast, the 725t plg-R displayed a slower rate of internalization of ligand compared with wild-type or \( \Delta 670-707 \) plg-R (see Fig. 6). The half-time for internalization for the 725t plg-R was approximately 2.0 min (40% of wild-type receptor). By comparison, the tail-minus receptor, as described previously (Mostov et al., 1986), demonstrates no detectable internalization of ligand in this assay (see Fig. 6). (We observed an appreciable amount of anti-SC Fab binding to the basolateral surface of MDCK cells which express the tail-minus receptor. Although approximately 90% of newly synthesized tail-minus receptor is delivered to the apical surface, 10% is delivered basolaterally. Since this receptor has no detectable internalization, the tail-minus receptor accumulates at the basolateral surface, accounting for the high level of ligand binding.) Thus, the cytoplasmic domain of the plg-R is critical for internalization of ligand, and the results obtained for 725t plg-R demonstrate that rapid internalization (but not all internalization) is impaired by deletion of the C-terminal 30 amino acids. However, neither delivery of newly synthesized receptor to the basolateral surface nor post-endocytotic delivery of ligand was affected by the 725t plg-R mutation (see below).

It should be noted that a cytoplasmic tyrosine is contained within the deleted segment of the 725t plg-R (Tyr-734). Since mutation of tyrosine residues in both the LDL receptor (Davis et al., 1987) and the mannose 6-phosphate receptor (Lobel et al., 1989) impairs the rapid internalization of ligand, we asked if Tyr 734 might be similarly involved in internalization. Using oligonucleotide-directed mutagenesis, we changed the tyrosine at position 734 to serine, producing a receptor termed Ser-734 plg-R. As indicated in Fig. 6, the time course of internalization for the Ser-734 plg-R is identical to the slow rate observed for 725t plg-R. In all other assays, the Ser-734 plg-R behaved as the 725t plg-R (see Figs. 2, 4, 5, 7, and 8 (data not shown)).

**Post-endocytotic Delivery of Ligand**—We have shown previously that, by using the basolateral proteolysis assay described above, a single cohort of internalized anti-SC Fab fragments can be isolated and its fate after endocytosis followed in MDCK cells that express the wild-type receptor (Breitfeld et al., 1989b). Using this assay, we have shown that anti-Fab fragments are transcytosed, but a significant fraction (45%) recycles to the basolateral medium. This assay involves several cooling steps and a protease treatment of the basolateral surface prior to the final incubation at 37 °C. In order to develop a simpler assay of ligand delivery after endocytosis, we have modified the ligand uptake assay developed by Goldstein and co-workers (1983) for the LDL-receptor system. MDCK cells expressing wild-type or mutant plg-R were allowed to endocytose ligand for a brief time (10 min) at 37 °C, washed quickly to remove nonendocytosed ligand, and then apical and basolateral medium samples were examined for the appearance of either intact or degraded ligand over the 120-min incubation. All steps were performed at 37 °C without any intervening cooling steps. Protease stripping of uninternalized ligand was not found to be necessary. Fig. 7A demonstrates that over 120 min, for wild-type receptor, 53% of endocytosed anti-SC Fab fragments is transcytosed, 20% returns to the basolateral medium, and only 6% is degraded and released into the apical and basolateral medium. The balance (21%) remains cell-associated after 120 min. Fig. 7B demonstrates the results of such an experiment with 725t plg-R and indicates that the fate of anti-SC Fab fragments endocytosed by the 725t plg-R is similar to wild-type receptor. However, the fate of ligand endocytosed by the \( \Delta 670-707 \) plg-R is quite different from wild-type (Fig. 7C). Only 7% of ligand is transcytosed into the apical medium intact. This represents 13% of the amount of transcytosis observed for wild-type receptor. Whereas 6% of ligand endocytosed by wild-type receptor is degraded and released into the medium, 50% of ligand endocytosed by the \( \Delta 670-707 \) plg-R is degraded and released (Fig. 7C). This degradation represents an 8-fold increase over that found with wild-type receptor. Like wild-type, 24% of ligand recycles to the basolateral medium intact. The balance (19%) remains cell associated after 120 min. Thus, post-endocytotic delivery of anti-SC Fab fragments is substantially altered when endocytosed by the \( \Delta 670-707 \) plg-

![FIG. 7. Fate of basolaterally endocytosed anti-SC Fab fragments. 125I-Labeled anti-SC Fab fragments (40 μg/ml) were endocytosed from the basolateral surface of MDCK cells expressing wild-type (A), 725t (B), or \( \Delta 670-707 \) plg-R (C) for 10 min at 37 °C, and the basolateral surface was washed four times quickly with medium. After washing, cells were placed in fresh wells with medium at 37 °C. The apical and basolateral medium was collected at the times indicated. To determine the amount of ligand that appeared intact in the apical (closed triangles) or basolateral medium (closed circles), or degraded and released into the medium (dashed line, open squares), samples were precipitated in trichloroacetic acid, and the soluble (degraded) and insoluble (intact) material was separated and counted in a γ counter. Each point represents the mean of duplicate filters from a typical experiment. MDCK cells not expressing the plg-R were analyzed in parallel and the values obtained for each time point were subtracted as nonspecific background. Values are expressed as a percentage of the total amount of ligand endocytosed during the initial 10-min incubation at 37 °C (MDCK, 535 cpm; wild-type, 11,087 cpm; 725t, 1,236 cpm; \( \Delta 670-707 \), 2356 cpm).](image-url)
R with a shift from the transcytotic pathway to one of degradation. Thus, deletion of residues 670–701 affects ligand delivery after endocytosis but does not affect basolateral delivery of receptor or the time course of internalization of receptor-bound ligand.

**Ligand Dissociation**—Are the altered phenotypes observed for our mutant plg-Rs due to alterations in receptor traffic produced by the mutations or simply due to altered rates of ligand dissociation from mutant receptor? For example, ligand may dissociate more rapidly from the 725t plg-R, and thus an apparent altered time course of internalization would be observed. Alternatively, internalized ligand may dissociate more rapidly from the A670–707 plg-R (especially in acidic intracellular compartments) and then ligand may be delivered by fluid phase to lysosomes for degradation. To address these issues, we measured the time course of dissociation of ligand (anti-SC Fab fragments) at 37 °C from the basolateral surface of cells expressing either wild-type or mutant receptor. To inhibit endocytosis of receptor-bound ligand we included 0.2 M sucrose in the medium. As observed by others (Heuser and Anderson, 1989), we found in preliminary experiments that this hypertonic treatment inhibited at least 85% of anti-SC Fab fragment internalization by wild-type or mutant plg-R. The time course of dissociation of anti-SC Fab fragments from receptor is virtually the same for wild-type and mutant plg-Rs (Fig. 8A).

If the mutant plg-Rs are grossly altered in structure, the rate of dissociation of the natural ligand (dIgA) might differ from wild-type. Therefore, we also investigated the time course of dissociation of dIgA from wild-type and mutant receptors. We found that the time course for dIgA dissociation was very similar for all forms of the receptor (Fig. 8B), suggesting that the structure of the ligand binding domain is not altered by any of our mutations. Of note, under these conditions, the rates of dissociation for anti-SC Fab fragments are substantially slower than for dIgA.

Since ligand might also dissociate intracellularly, particularly in acidic endosomes, we also measured rates of dissociation of ligand from receptor at pH 5.3 (which also inhibits endocytosis (Cosson et al., 1989)). At acidic pH, dissociation rates were similar for wild-type and mutant receptors with both anti-SC Fab fragments (Fig. 8C) and dIgA (Fig. 8D).

Thus, the altered time course of ligand internalization demonstrated for the 725t plg-R and the altered fate of ligand endocytosed by the A670–707 plg-R is unlikely to be the consequence of altered rates of dissociation of ligand from receptor.

Since ligand (either anti-SC Fab fragments or dIgA) does dissociate from receptor at an appreciable rate at 37 °C, determining the fate of the ligand is an imperfect way to follow the fate of the receptor. However, it is unlikely that the ligand is simply entering the fluid phase after endocytosis. First, the majority of a fluid phase marker endocytosed at the basolateral surface for 2 h at 4 °C, and then the basolateral surface was washed extensively. Cells were warmed to 37 °C, and the basolateral medium was sampled at the times indicated. Ligand released was determined by γ counting. MDCK cells that do not express the plg-R were analyzed in parallel, and these values were subtracted as nonspecific background. All points are the mean of duplicate filters.

**DISCUSSION**

The intracellular traffic of receptors and ligands is a central issue in cell biology. Previously we found that deleting 101 of the 103 amino acids of the cytoplasmic domain of the plg-R interfered with both basolateral delivery and with internalization of the receptor. In the current study we have found that deleting the C-terminal 30 residues yields a receptor which is internalized slowly, but otherwise follows the same pathway as wild-type receptor. A deletion of 38 residues from the middle of the cytoplasmic domain yields a receptor which is delivered to the basolateral surface and internalized almost as rapidly as wild-type receptor, but then is largely degraded instead of transcytosed. We have therefore demonstrated that different segments of the cytoplasmic domain of the plg-R, when deleted, affect different types of receptor and ligand traffic in polarized cells.
One explanation for these results is that these mutations grossly perturb the folding of the receptor, leading to aberrant traffic. The definitive method to investigate protein folding is x-ray crystallography. Other methods that have been used include binding of a panel of well defined monoclonal antibodies that are conformational specific (Copeland et al., 1988), sedimentation in sucrose gradients (Ng et al., 1989), changes in resistance to proteolysis and/or denaturation (Doms and Helenius, 1986), or various assays of function of the protein (Schlessinger, 1986). Since we can quantitatively assay several functions of the pig-R, we have used the latter approach. Unlike several other membrane proteins whose mutants may not reach the cell surface because they are not folded properly (e.g. HA and vesicular stomatitis virus G-protein (reviewed in Rose and Doms, 1988)), all of our mutant pig-Rs are rapidly and accurately delivered to the (basolateral) surface. Moreover, all bind ligand (both anti-SC Fab fragments and dIgA) and the rates of dissociation of ligand (at pH 7.4 and 5.3) are very similar to wild-type. By these functional criteria, the pig-R contains several structural determinants which act as cytoplasmic domain can produce different phenotypes. If types reported here.

For the Δ670-707 pig-R, misfolding could lead to artificial aggregation or oligomerization and thus cause mistargeting to lysosomes. Cross-linking of other receptors by antibodies can cause such misrouting (Mellman and Plutner, 1984). However, we have shown that cross-linking the wild-type pig-R with polyvalent antibodies against the receptor do not prevent it from being transcytosed (Mostov and Deitcher, 1986). Similarly, large polyvalent antigen-antibody complexes are also transcytosed by the pig-R (Brown et al., 1983). Finally, we have been unable to detect oligomers of the wild-type or mutant pig R's by chemical cross linking or by aqueous gradients at neutral and mildly acidic pH values. Thus, we think it is unlikely that our mutations have caused aberrant aggregation of the receptor.

Our most striking finding is that different mutations in the cytoplasmic domain can produce different phenotypes. If these mutations act by grossly altering the folding of the receptor, then at least three different artifactual conformations of the receptor must exist. One would correspond to the previously reported tail-minus receptor which is not basolaterally delivered, whereas the other conformations would correspond to the slow internalization and degradation phenotypes reported here.

Taken together these arguments make it unlikely that our results are simply due to misfolding of the receptor. Another possible explanation is that the cytoplasmic domain of the pig-R contains several structural determinants which act as independent sorting signals and may interact with proteins in the cytoplasm. This hypothesis can be tested by transferring the putative sorting signals to other proteins. What putative sorting signal could be deleted in the Δ670-707 pig-R mutant? The wild-type pig-R very efficiently avoids the cytoplasmic domain of a protein which is normally excluded from coated pits can induce its internalization via coated pits (Lazarovits and Roth, 1988).

Finally, we have found that if the entire cytoplasmic domain is deleted, the receptor is apically delivered, whereas if residues 670-707 or 726-755 are deleted, the receptor is basolaterally delivered. This suggests that either residues 653-669 and/or 708-725 are involved in basolateral delivery.

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