Transcytosis of the polymeric immunoglobulin receptor (pIgR) can be experimentally divided into three steps: 1) internalization from the basolateral plasma membrane and delivery to basolateral early endosomes, 2) microtubule-dependent movement from basolateral early endosomes to apical recycling endosomes, and 3) delivery from apical recycling endosomes to the apical surface and cleavage of the pIgR to secretory component, which is released into the apical medium. Transcytosis of the pIgR is stimulated by two signals, phosphorylation of Ser-664 in the cytoplasmic domain of the pIgR and binding of the ligand, dimeric IgA, to the pIgR. These signals do not detectably alter step 1 of transcytosis. Here, we show that phosphorylation of Ser-664 stimulates both steps 2 and 3, whereas binding of dimeric IgA stimulates only step 3 of transcytosis.

The plasma membrane of polarized epithelial cells is divided into separate apical and basolateral surfaces, which have very different protein and lipid compositions. Epithelial cells utilize two pathways to send plasma membrane proteins to the correct surface (Mostov et al., 1992; Rodriguez-Boulan and Powell, 1992). Newly made proteins traverse the secretory pathway to the trans-Golgi network (TGN), where they can be packaged into vesicles that deliver them directly to the correct surface. Alternatively, protein can be delivered first to one surface (generally the basolateral) and then endocytosed and transcytosed via vesicles to the apical surface. Transcytosis is the only pathway for delivery of membrane proteins to the apical surface that is universally found in all epithelial cells examined so far, and in some cell types it is the only pathway for delivery of membrane proteins to the apical surface (Mostov et al., 1992).

The polymeric immunoglobulin receptor (pIgR) has been a useful model system for studying polarized traffic from the TGN and transcytosis (Mostov, 1994). The pIgR has been expressed from cloned DNA in the Madin-Darby canine kidney (MDCK) cell line. When grown on a filter support, this cell line forms a well polarized, tight epithelial monolayer. The transfected pIgR functions as in vivo. The pIgR is sent first from the TGN to the basolateral surface, where it can bind its ligand, dimeric IgA (dIgA). With or without dIgA bound, the pIgR is then rapidly endocytosed and delivered to endosomes. The pIgR then moves through several vesicular compartments and is eventually exocytosed at the apical surface. At that surface, the extracellular ligand binding domain of the pIgR is proteolytically cleaved off and released together with the dIgA into extracellular secretions. This cleaved fragment of the pIgR is called secretory component (SC), and it protects the dIgA from proteolytic degradation.

Recent work has better defined the pathway taken by transcytosing pIgR and its bound ligand (Apodaca et al., 1994; Barr and Hubbard, 1993; Barroso and Sztul, 1994; Quintart et al., 1989). Transcytosis can be divided into three steps, each of which can be separately measured (Fig. 1). Step 1 is internalization at the basolateral plasma membrane and delivery to basolateral early endosomes that underly the basolateral plasma membrane.

In step 2, the dIgA is moved from the basolateral early endosomes to a tubulo-vesicular compartment that immediately underlies the center of the apical surface. This movement is blocked by the microtubule depolymerizing agent, nocodazole (Breitfeld et al., 1990; Hunziker et al., 1990). Recent work has uncovered several properties of this tubulovesicular compartment, which we have termed the apical recycling endosome (Apodaca et al., 1994; Barr and Hubbard, 1993; Barroso and Sztul, 1994; Hughson and Hopkins, 1990; Quintart et al., 1989). The apical recycling endosome contains transferrin, which is endocytosed via the transferrin receptor from the basolateral surface and which will return to the basolateral surface. Furthermore, the apical recycling endosome also receives membrane-bound (but largely not fluid phase) material, which is endocytosed from the apical compartment and which mainly recycles to the apical surface. For these reasons, we have suggested that the apical recycling endosome is the polarized cell equivalent of the recycling endosome that has been described in non-polarized cells. The apical recycling compartment appears to be a central location for sorting in the endosomal system, as it receives molecules from both surfaces and sorts them to the correct surface.

In step 3, the pIgR and bound ligand are delivered from the apical recycling compartment to the apical plasma membrane. The pIgR is then cleaved to SC, which is released into the apical medium. Several signals have been identified that control the sorting of the pIgR at different points in its pathway. A membrane-
proximal 17-residue segment of the cytoplasmic domain of the plgR is necessary and sufficient to direct the plgR (or a reporter molecule) from the TGN to the basolateral surface (Casanova et al., 1991). Systematic mutagenesis has identified 3 residues within this segment that are particularly important for basolateral targeting (Aroeti et al., 1993). Both in the TGN and in the endosomal pathway, the plgR undergoes polarized sorting that directs it to either the apical or basolateral surface. Mutations in the 17-residue basolateral sorting signal that decrease TGN to basolateral delivery and correspondingly increase TGN to apical delivery have a similar effect on sorting in the endosomal pathway (Aroeti and Mostov, 1994). That is, these mutations also decrease delivery from endosomes to the basolateral surface (recycling) and correspondingly increase delivery from endosomes to the apical surface (transcytosis). These data indicate that the same signal controls polarized sorting in the exocytic and endocytic pathways.

Once the plgR reaches the basolateral surface, its internalization (i.e. step 1 of transcytosis) is controlled by two internalization signals centered on Tyr-668 and Tyr-734 (Okamoto et al., 1994). These signals are quite similar to tyrosine-based internalization signals found in many other receptors. Rapid internalization is also regulated by phosphorylation of Ser-726, which is one of two major sites for phosphorylation of the plgR (Okamoto et al., 1994). Mutation of Ser-726 to an Ala (plgR-A726) decreases the rate of internalization of plgR by about two-thirds. This mutation does not significantly alter any other aspect of the trafficking of the plgR.

After internalization, steps 2 and/or 3 of transcytosis of the plgR are regulated by several signals. Transcytosis of dIgA and plgR is stimulated by activation of protein kinase C (Cardone et al., 1994) and by activation of the heterotrimeric G protein (Barroso and Sztul, 1994; Bomsel and Mostov, 1992, 1993; Hansen and Casanova, 1994a), which appears to act through cAMP and protein kinase A. These signaling mechanisms stimulate transcytosis of a variety of other molecules. For both protein kinase C and protein kinase A, it has been shown that a major effect is to increase movement of transcytosing molecules from the apical recycling endosomes to the apical plasma membrane, i.e. step 3 of transcytosis (Cardone et al., 1994; Hansen and Casanova, 1994a).

Transcytosis of the plgR is specifically stimulated by two other signals. Phosphorylation of Ser-664 in the cytoplasmic domain of the plgR (the other major site of phosphorylation) promotes transcytosis of the plgR, especially when no ligand is bound (Casanova et al., 1990). Mutation of Ser-664 to an Ala (plgR-A664) decreases transcytosis of the plgR and increases recycling to the basolateral surface. Mutation of Ser-664 to an Asp (plgR-D664), whose negative charge mimics a phospho-serine, increases transcytosis and decreases recycling. Our data are consistent with a model whereby the 17-residue basolateral signal first directs the plgR to the basolateral surface. At that surface or after endocytosis, phosphorylation of Ser-664 inactivates the basolateral signal, thereby enabling the plgR to be transcytosed. The signal of Ser-664 phosphorylation will even serve to promote transcytosis when transferred to a heterologous reporter molecule (Apodaca and Mostov, 1993). We previously constructed chimeric proteins containing the extracellular domain of such a reporter, placental alkaline phosphatase, fused to the membrane-spanning segment and cytoplasmic domain of the plgR. When Ser-664 of the construct was replaced by Ala, transcytosis of the chimera was decreased, while replacement of Ser-664 with Asp increased transcytosis.

Transcytosis plgR is also stimulated by binding of the ligand, dIgA. Hirt et al. (1993) first reported that transcytosis of plgR-A664 is stimulated by dIgA binding to the receptor. Subsequently, we reported that transcytosis of the wild-type plgR (plgR-WT) is stimulated by dIgA binding (Song et al., 1994). Although the signals of Ser-664 phosphorylation or dIgA binding can each stimulate transcytosis on their own, maximal stimulation of transcytosis is only observed when both signals are present.

The signals of Ser-664 phosphorylation and ligand binding both appear to be physiologically relevant. Stimulation by dIgA binding allows the transport of dIgA to be adjusted in response to the amount of dIgA presented to the cell. Phosphorylation of Ser-664 particularly promotes transcytosis of the empty plgR and subsequent production of SC without IgA bound ("free SC"). Most secretions contain significant amounts of free SC, which may function by mass action to increase the fraction of dIgA that is bound to SC and thereby protected from proteases (Song et al., 1994).

We have previously determined that the signals of Ser-664 phosphorylation and dIgA binding do not alter step 1 of transcytosis, i.e. internalization (Song et al., 1994). However, it is not known whether these signals stimulate transcytosis by increasing step 2 and/or step 3 of transcytosis. In this paper, we investigate which of these two steps is affected by each of these signals.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human dIgA was kindly provided by J. P. Vaerman. This material consisted of approximately 70% dimer, 20% trimer, and 10% tetramer and was shown to be transcytosed and stimulate plgR transcytosis as effectivley as highly purified dimeric IgA. Avidin-horseradish peroxidase was from Vector Laboratories (Burlingame, CA). All other materials were from Sigma or Fisher.

**Cell Culture**—MDCK strain II cells expressing the plgR-WT or plgR-A664 were maintained in MEM with 10% fetal bovine serum and antibiotics as described (Mostov and Deitcher, 1986). For all experiments, cells were cultured on 12-mm diameter, 0.4-micron pore size Transwell units as described. The cells were fed every other day and used 3–5 days postculture.

**Assay for Step 2 of plgR Transcytosis**—We used a previously described modification of the diaminobenzidine (DAB) cross-linking procedure (Apodaca et al., 1994). MDCK cells on Transwells were rinsed in Hank’s balanced salt solution containing 25 mM Heps, pH 7.4 (HBSS), and then biotinylated from both the basolateral and apical surfaces with 0.2 mg/ml sulfo-L-cysteine(6-biotinamido) hexaazopropylsulfonic acid (SAHS) at 15 °C for 15 min (Song et al., 1994). The biotinylation was repeated with a freshly made solution of sulfo-L-cysteine(6-biotinamido) hexaazopropylsulfonic acid for a second period of 15 min. Excess biotin was then quenched with MEM-BSA (MEM with Hank’s salts, 2.5 g/liter NaHCO₃, 20 mM Heps-Na, pH 7.4, 6 mg/ml BSA, and antibiotics) at 15 °C. Ligands were then internalized at 15 °C for 20 min after the quenching of the biotinylation. The apical ligand was avidin-horseradish peroxidase, 25 µg/ml in a volume of 0.2 ml. Where indicated, the basolateral ligand of dIgA (10.5 µg/ml) was included. The basolateral surface of the filter was placed on a 20-µl drop of MEM-BSA dIgA. The cells were then chased for 10 min at 37 °C in the presence of avidin-horseradish peroxidase (25...
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pg/ml and trypsin (25 μg/ml) in the apical medium and the presence or absence of dIgA (0.3 mg/ml) in the basolateral medium. After the 37 °C chase, the trypsin was quenched with 15% horse serum in MEM-BSA at 4 °C, and the cells were then washed three times with ice-cold HBSS.

Freshly made and filtered DAB reaction buffer (0.5 mg/ml DAB in HBSS, pH 7.4, 0.03% H2O2, w/v) was added to both the apical and basolateral chambers of the Transwell at 4 °C and incubated for 45 min. In control reactions, the H2O2 was omitted. Afterward, the cells were washed twice with HBSS at 4 °C. The filters were excised from the Transwell holder with a scalpel, boiled for 100 s in 0.5 ml of SDS lysis buffer (0.5% SDS (w/v), 100 mM triethanolamine-HCl, pH 8.6, 5 mM EDTA, 0.02% Na3VO4, w/v) and vortexed for 15 min at 4 °C. The lysates were then centrifuged at 100,000 × g in a Sorvall RP70AT rotor for 25 min at 4 °C, and the supernatants were diluted with Triton X-100 and immunoprecipitated with a sheep antiserum to rabbit SC (Casanova et al., 1991). The immunoprecipitates were analyzed by SDS-PAGE and visualized by streptavidin-horseradish peroxidase and ECL and quantitated with a Molecular Dynamics densitometer.

RESULTS

We have previously developed an assay for delivery of transepithelial dIgA from the basolateral early endosome to the apical recycling endosome (step 2) (Apodaca et al., 1994). In the original assay, we followed radiiodinated dIgA that had been internalized from the basolateral surface. Here, we wished to examine the effect of dIgA binding on the movement of the pIgR itself. Therefore, we used a method that we previously described of labeling the pIgR at the basolateral surface with biotin (Song et al., 1994) and combined this with our method for measuring step 2.

Both surfaces of MDCK cells expressing either pIgR-WT or pIgR-A664 were biotinylated for a total of 30 min at 18.5 °C, which created pools of biotinylated proteins (including biotin-pIgR) on both the basolateral and apical surfaces and in endosomes (Fig. 2, step 1). Next, the cells were exposed to avidin-horseradish peroxidase at the apical surface at 18.5 °C for 10 min. In some cases, the cells were simultaneously exposed to dIgA at the basolateral surface (Fig. 2, step 2). We and others (Apodaca et al., 1994; Barroso and Szot, 1994; Hunziker et al., 1990) have previously shown that at 18.5 °C the pIgR is able to internalize, recycle to the basolateral surface, and move from the basolateral early endosomes to the apical recycling compartment, but the rates of these processes are much slower than at 37 °C. After a total of 40 min at 18.5 °C, the pIgR stays mainly in the basolateral endosome, as assessed by morphological and biochemical criteria (Apodaca et al., 1994; see below). Similarly, apical membrane proteins recycle between the apical surface and the apical endosome at 18.5 °C. Therefore, we expect that during the incubation at 18.5 °C, avidin-horseradish peroxidase would bind to biotinylated membrane proteins at the apical surface and start to fill up the apical recycling endosome. When the basolateral surface of cells were exposed to dIgA for 10 min after biotinylation, some of the biotin-pIgR molecules would bind to the dIgA and be internalized.

The cells were then chased for 10 min at 37 °C to allow biotinylated pIgR to move from the basolateral endosome to the apical recycling endosome (Fig. 2, step 3). We had previously shown that >80% of basolaterally internalized dIgA could move to the apical recycling endosome in 10 min at 37 °C, so this is an appropriate chase period (Apodaca et al., 1994). During this 37 °C chase, avidin-horseradish peroxidase was continuously present in the apical medium, while (where indicated) dIgA was continuously present in the basolateral medium. Trypsin was included in the apical medium to digest pIgR that had reached the apical surface and prevent the pIgR from recycling back to the apical recycling compartment. We have previously shown that pIgR on the apical surface is rapidly and completely cleaved by trypsin under these conditions (Apodaca et al., 1994). After the 37 °C chase, the intact cells were treated with 4 °C with the DAB and H2O2, which are membrane permeable (Fig. 2, step 4). As previously shown, the biotin-pIgR that had reached the avidin-horseradish peroxidase-filled apical recycling endosome was cross-linked by the DAB reaction product into a dense, detergent-insoluble complex (Apodaca et al., 1994). The cells were then lysed with SDS, and the insoluble
Material was removed by centrifugation (Fig. 2, step 5). The biotin-pIgR in non-horseradish peroxidase containing compartments of the cell remained soluble and was purified by immunoprecipitation, analyzed by SDS-PAGE, blotted onto a filter, detected by probing with streptavidin-horseradish peroxidase and enhanced chemiluminescence, and quantitated with a densitometer. Therefore, the percentage of the biotin-pIgR in the apical recycling endosome is computed by subtracting the biotin-pIgR left in the cell after the DAB/H$_2$O$_2$ reaction from the total biotin-pIgR in control cells that were not reacted with DAB/H$_2$O$_2$.

As shown in Fig. 3, after the 37°C chase, 62.5 ± 2.4% of the biotin-pIgR-WT was in the apical endosome. When the 10-min chase at 37°C was omitted, only 18.6 ± 1.2% of the biotin-pIgR-WT was in the apical endosome (Fig. 4), which is consistent with our previous data that relatively little movement to the apical recycling endosome occurs during 30 min at 18.5°C (Apodaca et al., 1994). Note, however, that omission of the 10-min chase at 37°C might also reduce the amount of avidin-horseradish peroxidase internalized from the apical surface. When we used the pIgR-A664, we found that 40.1 ± 2.3% of the biotin-pIgR-A664 reached the apical recycling endosome after the 37°C chase. This was 30% less than the pIgR-WT in an otherwise identical experiment; this difference was statistically significant ($p < 0.05$). These data indicate that the movement of pIgR-A664 from the basolateral early endosome to the apical recycling endosome (step 2) is slower than that of pIgR-WT. This suggests that phosphorylation of Ser-664 stimulates step 2 of transcytosis.

These experiments were performed in the absence of dlgA. As shown in Fig. 3, addition of dlgA to the basolateral medium during both the 18.5°C pulse and the subsequent 37°C chase produced no detectable change in the results. This suggests that dlgA does not detectably stimulate step 2 of transcytosis of either pIgR-WT or pIgR-A664.

Step 2 of transcytosis is known to be inhibited by depolymerization of microtubules by nocodazole at 4°C (Apodaca et al., 1994; Barroso and Sztul, 1994; Breitfeld et al., 1990; Hunziker et al., 1990). Therefore, as a control we repeated the experiment with cells that had been pretreated with nocodazole. As shown in Fig. 4, 20.2 ± 2.8% of the biotin-pIgR-WT was in the apical recycling compartment, i.e., a 3-fold reduction from the experiment without nocodazole. This indicates that the process that we have measured is largely dependent on microtubules. This control documents that the horseradish peroxidase reaction is truly measuring delivery of pIgR to the apical recycling endosome and that the insolubilization of the pIgR is not due to some other nonspecific process. Inclusion of dlgA in the nocodazole experiment produced no significant effect (Fig. 4). Note that omission of the 10-min chase at 37°C gave a similar result to the nocodazole treatment, suggesting that the distribution of pIgR at the end of the 18.5°C incubation was comparable with that in nocodazole-treated cells, i.e., that the pIgR had largely not reached the apical recycling compartment.

Next, we examined the effects of dlgA and Ser-664 phosphorylation on step 3 of pIgR transcytosis, delivery of pIgR from the apical recycling endosome to the apical plasma membrane. Our assay is diagramed in Fig. 5. The pIgR at the basolateral surface was biotinylated and internalized at 18.5°C (Fig. 5, step 1), and in some cases dlgA was bound to the pIgR and internalized at 18.5°C (Fig. 5, step 2). The cells were then chased at 37°C (Fig. 5, step 3). As shown above, ~60% of pIgR-WT and ~40% of pIgR-A664 had reached the apical recycling endosome after the 18.5°C incubations and the 10-min chase at 37°C. To increase the signal of biotin-pIgR in the apical recycling endosome, we lengthened this 37°C chase to 15 min. After allowing the receptor to reach the apical recycling endosome during this chase, the cells were then treated with 33 μM nocodazole at 4°C to disassemble microtubules; this inhibited further delivery of pIgR to the apical recycling compartment (Fig. 5, step 4). The cells were subsequently chased a second time for 20 min at 37°C in the continued presence of nocodazole (Fig. 5, step 5). As previously shown, nocodazole does not block delivery from the apical recycling compartment to the apical plasma membrane. Trypsin was included in the apical medium to prevent reinternalization of pIgR from the apical surface, thereby allowing us to focus solely on initial delivery to the apical surface (Apodaca et al., 1994). The biotin-
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**FIG. 5. Schematic diagram of the assay for measuring step 3 of transcytosis.** b-plgR, biotin-plgR.

1. Biotinylate basolateral surface for 30 min at 18°C. Biotin-plgR is internalized.

2. In some cases dIgA is bound to the plgR and internalized for 10 min 18°C. For clarity dIgA is not shown in subsequent frames.

3. Chase 15 min at 37°C to allow plgR to reach apical recycling endosome.

4. Depolymerize microtubules with nocodazole at 4°C to prevent further transport to apical recycling endosome.

5. Second chase for 20 min at 37°C to allow plgR to reach apical surface and be cleaved to SC. Measure b-plgR remaining in cells.

plgR left in the cells was immunoprecipitated, resolved by SDS-PAGE, blotted, detected by streptavidin horseradish peroxidase and ECL, and quantitated with a densitometer. In control cells the biotin-plgR was allowed to reach the apical recycling endosome, i.e. the cells were carried through the first 37°C incubation, as well as the nocodazole treatment at 4°C. However, the second 37°C incubation (Fig. 5, step 5) was omitted. We computed the percentage of the biotin-plgR, which reached the apical surface during the second 37°C chase as the fraction of biotin-plgR lost from the experimental cells relative to the control cells.

As shown in Fig. 6, in the absence of dIgA, 63.1 ± 2.4% of biotin-plgR-WT and 50.4 ± 2.0% of biotin-plgR-A664 reached the apical plasma membrane during the 20 min of the second 37°C chase. Statistically, these values were significantly different (p < 0.05). Therefore, plgR-A664 was slower than plgR-WT in this step, indicating that the mutation of Ser-664 to Ala inhibits step 3 of plgR transcytosis.

The experiments in the preceding paragraph were performed in the absence of dIgA. When dIgA was included in the ligand pulse (10 min at 18.5°C) and the first 37°C chase, the percentage of the biotin-plgR that reached the apical surface increased from 63.1% to 71.0 ± 1.7% for plgR-WT and from 50.4% to 61.8 ± 0.8% (Fig. 7). These increases were statistically significant (p < 0.05 in both cases), indicating that dIgA stimulates step 3 of transcytosis for both plgR-WT and plgR-A664.

A possible confounding factor was that the presence or absence of dIgA could change the amount of plgR in the cells at the starting point, i.e. at the beginning of the second 37°C chase. We therefore compared the total amount of biotin-plgR (-WT or -A664) present in the cells at this point in the presence or absence of dIgA and did not find any difference (Fig. 7). This is in agreement with the data in Fig. 3 that dIgA has no effect on the delivery of plgR to the apical recycling endosome.

**DISCUSSION**

Transcytosis of the plgR can be experimentally divided into at least three steps: 1) internalization into basolateral early endosomes, 2) delivery from basolateral early endosomes to
been shown that step 1 is regulated by phosphorylation of Ser-726 (Okamoto et al., 1994). Step 3 is regulated by protein kinase C and cAMP (Cardone et al., 1994; Hansen and Casanova, 1994a), but these intracellular signals act to generally stimulate step 3 for a variety of molecules, not just the pIgR. Here, we have investigated which steps are regulated by two other signals, i.e. phosphorylation of Ser-664 and binding of dIgA to the pIgR. The data presented here indicate that mutation of Ser-664 to Ala reduces both steps 2 and 3. This suggests that phosphorylation of this Ser stimulates both of these steps. In contrast, binding of dIgA stimulates step 3 but does not detectably stimulate step 2.

The various stimulatory effects observed here are small but quite reproducible and statistically significant. There are several additional reasons to think that our results are biologically significant and that the measurements may underestimate stimulation. First, the observation that Ser-664 phosphorylation affected both steps 2 and 3, whereas dIgA binding affected only step 3, confirms that our measurements are capable of distinguishing effects on these two steps. In contrast, if we had found that both Ser-664 phosphorylation and dIgA binding appeared to affect both steps, we could not be sure that our assays could reliably distinguish effects on one step or the other.

Second, we have previously observed that dIgA bound to the pIgR is transcytosed several-fold faster than is biotin-pIgR in the presence of dIgA (Song et al., 1994). This is most likely due to the failure of many molecules of biotin-pIgR to bind to the dIgA, which was added only after the biotin reaction was completed. Ideally, we would have preferred to include the dIgA in the biotinylation solution at the basolateral surface, which might increase the fraction of biotinylated pIgR molecules that bound pIgR. However, exposure of the dIgA to the high concentration of sulfo-succinimidyl-6-(biotinamido) hexanoate used to derivatize the cell surface destroyed the ability of the dIgA to bind to pIgR (Song et al., 1994). Therefore, we were required to use a procedure where dIgA was added only after biotinylation was complete. It is likely that many of the biotin-pIgR molecules were endocytosed before addition of the dIgA and thus did not have the opportunity to bind to dIgA; this probably led to an underestimate of the effect of dIgA. We also tried an alternative procedure of biotinylation of the pIgR at the basolateral surface at 4°C, followed by washing and then binding of dIgA at this temperature. However, the signal of biotinylated pIgR was too small to reliably quantitate, probably because fewer molecules were biotinylated at 4°C compared with 18.5°C.

Third, we have used various experimental manipulations, such as low temperature incubations and nocodazole treatment, to trap the pIgR in various compartments. In every case, we chose a specific time for each incubation. All of these conditions were chosen after a large number of trials, varying incubation times, temperatures, and other parameters. A major criterion for selecting the final conditions used was reproducibility. However, under any given conditions, we may miss a considerable portion of the stimulation that we wish to detect. For instance, if in the assay for delivery from the apical recycling endosome to the apical surface, we used a second chase at 37°C of only 10 min instead of 20 min, we could observe a more dramatic effect of the presence of dIgA. However, in preliminary experiments, these results were much more variable, probably because of the difficulty in reproducibly manipulating a large number of filters on a tight experimental schedule. Hence, our protocols are a compromise between maximizing differences and reproducibility.

Fourth, nocodazole and cold were used to selectively block step 2. However, microtubule depolymerization may have a subtle effect on step 3. The apical recycling endosome is normally clustered around the centriole in the center of the apical region of the cell. Treatment with nocodazole causes the apical recycling endosome to disperse throughout the apical cytoplasm (Apodaca et al., 1994).

Finally, our results are in agreement with our previous observations on the stimulation of transcytosis by dIgA (Song et al., 1994). Earlier, we found that 1) in the absence of dIgA, transcytosis of pIgR-A664 is slower than pIgR-WT and 2) dIgA produces a relatively small stimulation of transcytosis of pIgR-WT and a greater stimulation of transcytosis of pIgR-A664. These data are consistent with our present findings that the pIgR-A664 mutant is slower than pIgR-WT in both steps 2 and 3 of transcytosis and that dIgA binding produces a larger stimulation of step 3 for the pIgR-A664 than for the pIgR-WT.

Our conclusions on the sites of regulation of transcytosis by phosphorylation and dIgA binding are consistent with other recently developed concepts about the regulation of polarized membrane traffic of other molecules and are therefore likely to be of general interest. We have suggested that the pIgR contains a basolateral signal that directs the pIgR from the TGN to the basolateral surface and from the endosomal pathway back to the basolateral surface. This signal may be viewed as a basolateral retrieval signal that can direct the repeated recycling of endocytosed pIgR to the basolateral surface, analogous to retrieval signals operating in the secretory pathway (Pelham, 1991). The apparent role of Ser-664 phosphorylation is to inactivate this signal, thereby causing transcytosis (Aroeti and Mostov, 1994). Other basolateral recycling molecules are also thought to have similar basolateral retrieval signals. For instance, mutational inactivation of one of the basolateral signals of the low-density lipoprotein receptor causes this receptor to be transcytosed from the basolateral to the apical surface (Matter et al., 1993). We have suggested that for receptors that efficiently recycle to the basolateral surface, a portion of the recycling occurs directly from the basolateral early endosome to the basolateral surface, while some of the molecules reach the apical recycling endosome before recycling to the basolateral surface (Apodaca et al., 1994). This would explain how transferrin reaches the apical recycling endosome. We suggest that the pIgR-A664 is similar to these recycling receptors. Specifically, the pIgR is partially recycled from the basolateral early endosome to the basolateral surface and partially recycled from the apical recycling endosome to the basolateral surface. This is consistent with the observed decreases in both steps 2 and 3 of pIgR-A664 relative to pIgR-WT. Note that we assume that the principal effect of the mutation of Ser-664 to Ala is to prevent phosphorylation. It is possible that the mutation has other effects, e.g. on the conformation of the cytoplasmic domain.

In contrast, delivery of pIgR and many other kinds of molecules from the apical recycling compartment to the apical surface is regulated by several second messenger systems. For instance, activation of protein kinase C stimulates this step for pIgR and transferrin (Cardone et al., 1994), while activation of protein kinase A by cAMP stimulates this step for pIgR and ricin (Hansen and Casanova, 1994). Moreover, it has been suggested that the apical recycling compartment may be homologous to compartments that exhibit regulated delivery to the surface in other cell types, both epithelial (Harris et al., 1991) and non-epithelial. Viewed from this perspective, the stimulation of step 3 by dIgA binding is another example of this type of regulated surface delivery. This suggests that this stimulation by dIgA binding may be mediated by well established second messenger pathways.
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