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Stimulation of transcytosis of the polymeric immunoglobulin receptor by dimeric IgA

(phosphorylation/biotinylation/endosomes)

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ABSTRACT **The polymeric immunoglobulin receptor (pIgR) is transcytosed from the basolateral to the apical surface of polarized epithelial cells. We have previously shown that phosphorylation of Ser-664 in the cytoplasmic domain of the pIgR is a signal for its transcytosis. We now report that binding of a physiological ligand, dimeric IgA, to pIgR stimulates pIgR transcytosis. This stimulation occurs in both the presence or absence of Ser-664 phosphorylation. We have used three methods to measure transcytosis of the pIgR. (*i*) The pIgR was biosynthetically labeled and its cleavage to secretory component after transcytosis was measured. (*ii*) The pIgR was labeled with biotin at the basolateral surface. After transcytosis, release of the biotin-labeled secretory component into the apical medium was measured. (*iii*) Transcytosis of a ligand bound to the pIgR was measured. All three methods indicated that dimeric IgA stimulates transcytosis of the pIgR.**

Polarized epithelial cells have separate apical and basolateral surfaces with different compositions (1–3). To maintain these differences proteins and lipids must be continually sorted. Newly made plasma membrane proteins can be sorted in the trans-Golgi network into vesicles that transport them to the basolateral or apical surface. Upon reaching the plasma membrane many proteins can be endocytosed. In endosomes proteins can be sorted for recycling to the original cell surface or transcytosis to the opposite cell surface. As a model system to study this sorting, we have used the polymeric immunoglobulin receptor (pIgR). In many epithelial cells the pIgR is synthesized in the rough endoplasmic reticulum and sent to the Golgi apparatus and then the trans-Golgi network. From there it is targeted to the basolateral surface, endocytosed, and transcytosed to the apical surface (4). Upon reaching the apical surface, the extracellular, ligand-binding portion of the pIgR is proteolytically cleaved and released into apical secretions. This cleaved fragment is termed secretory component (SC). Ser-664 in the cytoplasmic domain of the pIgR is a major site for phosphorylation, and this phosphorylation is a signal for transcytosis (5, 6). Mutation of this serine to an alanine (pIgR-Ala^{664}) reduces the rate of transcytosis of the pIgR. Mutation of this serine instead to an aspartate (pIgR-Asp^{664}), in which the negative charge may mimic a phosphate, increases the rate of transcytosis.

A major function of the pIgR is to transcytose its ligand, dimeric IgA (dIgA), from the basolateral to the apical surface of a variety of epithelial cells (4, 6). In many secretions there is an excess of free SC (i.e., not bound to dIgA) over dIgA (7). This is especially true in IgA-deficient humans, who often have near-normal levels of free SC in their secretions (8). This free SC must be derived from pIgR that was transcytosed without bound ligand. Similarly, in perfused rat liver SC is released into bile in the absence of dIgA (9, 10). Transcytosis of pIgR has also been investigated in the polarized Madin–Darby canine kidney (MDCK) cell line. We expressed cloned rabbit pIgR in these cells and found that this pIgR is transcytosed in the absence of dIgA (11). These observations suggested that transcytosis of pIgR may be independent of ligand binding. We have now re-examined the dependence of pIgR transcytosis on ligand binding in MDCK cells and have found that binding of dIgA to pIgR stimulates transcytosis of the pIgR. The dIgA stimulates transcytosis of both the wild-type pIgR (pIgR-WT) and pIgR-Ala^{664}.

MATERIALS AND METHODS

Most methods have been published (12), and further details are given in the figure legends. Fetal bovine serum was from HyClone. Radioactive compounds were from Amersham. Other chemicals were from Sigma or Fisher. Iodinations were by the iodine monochloride method (12). Fab fragments of guinea pig antibodies against rabbit SC (12, 13) and various preparations of human dIgA were prepared as described (14, 15).

For pulse-chase analysis, cells were cultured on 6.5-mm-diameter Transwells (Costar) for 4 days in Eagle's minimal essential medium (MEM)/5% fetal bovine serum (FBS)/antibiotics (12). Cells were labeled on 10-μl drops of cysteine-free MEM containing [^{35}S]cysteine at 1 mCi/ml (1 Ci = 37 GBq) for 15 min. Transwells were rinsed and chased in MEM/BSA (MEM with Hanks' salts, 20 mM NaHepes at pH 7.4, BSA at 6 mg/ml, and antibiotics) at 37°C for various chase times. In some cases dIgA at 0.3 mg/ml was included in the basolateral chase medium. SC was immunoprecipitated from the apical and basolateral medium, and pIgR was immunoprecipitated from the cells at the end of the chase. Immunoprecipitates were analyzed by SDS/PAGE, and radioactivity was determined with a Molecular Dynamics PhosphorImager. Cumulative radioactivity in apical SC is plotted as a percentage of total initial radioactivity in pIgR. Radioactivity is in arbitrary units generated by the PhosphorImager.

Labeling for the assay of transcytosis of biotinylated pIgR was similar except that (*i*) larger Transwells (24 mm) were used. (*ii*) Cells were starved in cysteine-free medium for 15 min. (*iii*) Cells were labeled for 45 min by using a 50-μl drop

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Abbreviations: pIgR, polymeric immunoglobulin receptor; pIgR-WT, pIgR-Ala^{664}, or pIgR-Asp^{664}, pIgR with Ser-664, Ala-664, or Asp-664, respectively; SC, secretory component; dIgA, dimeric IgA; MDCK, Madin–Darby canine kidney; Tf, canine transferrin; BSA, bovine serum albumin.

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of medium in contact with the basolateral surface. Cells were chased for 30 min at 37°C, and then washed three times at 18°C with Hanks' buffer lacking phosphate and containing 20 mM NaHepes, pH 7.4. The basolateral surface was then incubated with this buffer containing sulfosuccinimidyl-6-(biotinamido)hexanoate at 0.1 mg/ml for two 15-min periods at 18°C (16). During the biotinylation reaction the apical surface of the cells was in contact with MEM/BSA. After biotinylation, both sides of the cells were washed three times quickly with the Hanks' buffer, once quickly with MEM/BSA, and then once with MEM/BSA for 10 min. All washes were at 18°C. Cells were then incubated for 35 min at 18°C in MEM/BSA with or without dIgA at 0.3 mg/ml in the basolateral medium. (Including dIgA during the biotinylation or washes did not yield any stimulation because the ability of dIgA to bind to the pIgR is damaged by biotinylation of the dIgA; data not shown.) Cells were washed twice quickly and once for 3 min with MEM/BSA. The cells were chased at 37°C for 30, 60, or 120 min in MEM/BSA. Cells and media were collected and processed for immunoprecipitation (12). Immunoprecipitated proteins were released from the protein G-Sepharose beads by boiling the beads with 20 μl of 10% SDS for 5 min. One milliliter of 2.5% Triton dilution buffer (see ref. 12) was then added, and the samples were centrifuged at 14,000 × *g* for 5 min. The supernatant was transferred to a new 1.5-ml tube, and 15 μl of a 50% slurry of streptavidin–agarose was added. The samples were rotated for 1–2 hr at room temperature, and the beads were then washed (12). Bound proteins were then analyzed by SDS/PAGE and quantitated with a PhosphorImager.

For the ligand transcytosis assays, 6.5-mm Transwells were placed on 10-μl drops containing radioactive ligand [^{125}I-labeled dIgA (^{125}I-dIgA), 10 μg/ml, 4 × 10^6 cpm/μg; or ^{125}I-labeled Fab (^{125}I-Fab), 4 μg/ml, 4 × 10^6 cpm/μg], and ligand was internalized for 10 min at 37°C. The Transwells were washed at 37°C four times over a 5-min total period with MEM/BSA. The Transwells were transferred to a 24-well culture plate, and fresh MEM/BSA was added to both the apical (200 μl) and basolateral (400 μl) chambers. This MEM/BSA was replaced after 7.5, 15, 30, 60, and 90 min. At the end of the 90-min chase at 37°C, radioactivity in all fractions was determined, including in the cells on the filter.

RESULTS

To examine the effect of dIgA on transcytosis, we have biosynthetically labeled the pIgR with a pulse of [^{35}S]cysteine and then followed its entire pathway, which leads ultimately to cleavage and release of SC in the apical medium (12). As described (5) ≈77% of the labeled pIgR was released as SC into the apical medium over a 5-hr chase (Fig. 1). If 0.9 μM dIgA (0.3 mg/ml) was included in the basolateral medium during the chase, the amount of SC released over 5 hr was increased to 89%, suggesting that dIgA increased pIgR transcytosis. This increase was statistically significant but small enough to have escaped previous notice.

Mutation of a major site of phosphorylation of the pIgR, Ser-664 → Ala (pIgR-Ala^{664}) profoundly decreased release of apical SC (Fig. 1) (5). If, however, dIgA at 0.3 mg/ml is included in the basolateral medium during the 5-hr chase, apical release of SC over 5 hr is increased from 30% to 66% (Fig. 1). This result indicates that binding of dIgA can stimulate transcytosis, even in the absence of phosphorylation of Ser-664.

The pulse-chase procedure followed the entire pathway of the receptor. To examine just transcytosis, the pIgR was metabolically labeled and chased to allow it to reach the basolateral surface (5, 12). Molecules at this surface were then biotinylated. After a second chase period, we then detected biotinylated SC in the apical medium, which gave a measure of transcytosis (Fig. 2). In agreement with previous results, release of apical SC was substantially less when pIgR-Ala^{664} was used. Including dIgA at 0.3 mg/ml in the basolateral medium increased SC release, especially when the pIgR-Ala^{664} was used. These data confirm that dIgA binding stimulated transcytosis of the pIgR and that this stimulation occurred even when phosphorylation of Ser-664 was blocked.

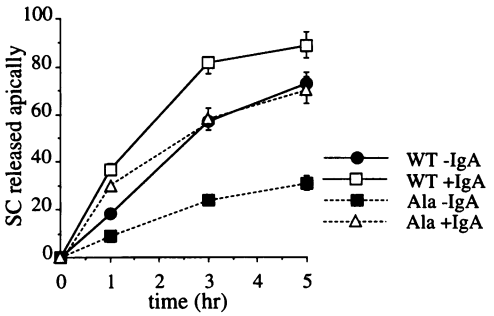

FIG. 1. dIgA stimulates release of metabolically labeled SC into apical medium. Cells expressing the pIgR-WT (WT) or pIgR-Ala^{664} (Ala) were metabolically labeled for 15 min and chased for various times. SC was immunoprecipitated from the medium, and pIgR was immunoprecipitated from the cells. Where indicated, dIgA at 0.3 mg/ml was included in the basolateral chase medium. Data are cumulative radioactive SC released into the apical medium, expressed as a percentage of total initial pIgR. Error bars are SEM and are often smaller than the plotting symbol (n = 6). The − IgA control data are repetitions of published work (5).

Previously we measured transcytosis of preinternalized ligand to monitor just the transcytotic leg of the pIgR pathway. We have used both the physiological ligand, ^{125}I-dIgA, and a pseudoligand, ^{125}I-Fab fragments of affinity-purified guinea pig antibodies against rabbit SC (16). To assay for transcytosis we preinternalized either ligand from the basolateral surface for 10 min at 37°C and then washed cells for 5 min at 37°C to remove surface-bound material. At this point there was a pool of preinternalized ligand. Next, the appearance of ligand in the apical medium was monitored during a 90-min chase period at 37°C. Fig. 3*A* shows that both the initial rate and final extent of transcytosis of ^{125}I-dIgA by the pIgR-WT were much greater than for ^{125}I-Fab. Mixing unlabeled dIgA (0.3 mg/ml) with the ^{125}I-Fab also increased transcytosis of ^{125}I-Fab, indicating that dIgA can at least partially promote transcytosis of ^{125}I-Fab bound to the pIgR and that the ^{125}I-Fab is not inhibiting transcytosis.

We have reported (5) that pIgR-Ala^{664} transcytoses ^{125}I-Fab poorly. In contrast, this mutant transcytoses ^{125}I-dIgA far better than ^{125}I-Fab (Fig. 3*B*). Furthermore, unlabeled dIgA (0.3 mg/ml) stimulates transcytosis of ^{125}I-Fab by the pIgR-Ala^{664} (Fig. 3*B*). These data are consistent with the hypothesis

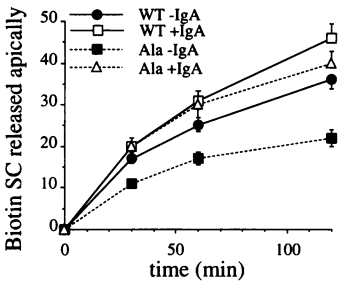

FIG. 2. dIgA stimulates the release of biotin-labeled SC into the apical medium from cells expressing the pIgR-WT (WT) or pIgR-Ala^{664} (Ala). Data are cumulative radioactive biotinylated SC released into the apical medium, expressed as a percentage of total initial radioactive biotinylated pIgR (n = 5).

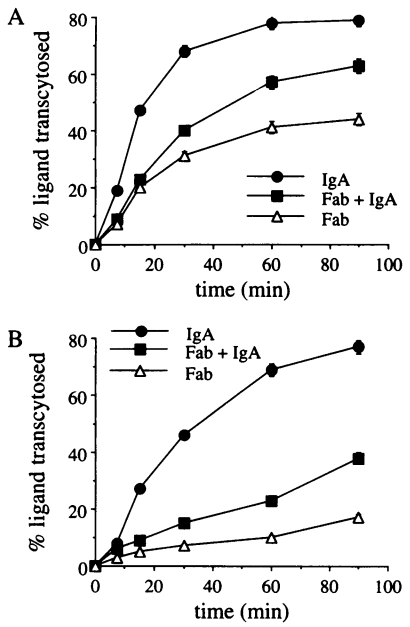

FIG. 3. Dimeric IgA is transcytosed faster than Fab. Cells expressing pIgR-WT (*A*) or pIgR-Ala^{664} (*B*) were used in an assay of transcytosis of preinternalized ligand. In some cases, unlabeled dIgA was premixed with the ^{125}I-Fab (Fab + IgA). The cumulative appearance of labeled ligand in the apical medium (i.e., transcytosed ligand) is plotted as a percentage of total ligand in all fractions (n = 6–12). Total cpm ranged from 3 to 5 × 10^3 for ^{125}I-dIgA and 8 to 12 × 10^3 for ^{125}I-Fab.

that dIgA binding stimulates transcytosis. The ^{125}I-Fab seem to follow the empty pIgR, as their transcytosis is much slower than the ^{125}I-dIgA. Moreover, although transcytosis of ^{125}I-dIgA was only slightly slowed by the pIgR-Ala^{664} mutation, transcytosis of the ^{125}I-Fab (like transcytosis of the empty receptor itself) was much more slowed by this mutation.

Transcytosis of ligands, particularly ^{125}I-dIgA, was much faster than transcytosis of the pIgR itself. There are at least two nonmutually exclusive explanations. (*i*) With ^{125}I-dIgA we follow only those molecules of pIgR that have actually bound dIgA, whereas in the pulse-chase and biotin-SC methods we measure the entire population of pIgR. We found that dIgA at 0.3 mg/ml gave a maximal stimulation of transcytosis in the pulse-chase assay; no further effect was seen with [dIgA] up to 0.9 mg/ml. However, given that endocytosis of pIgR has a $t_{1/2}$ of 1 min (5, 17) and that much of the basolateral surface may be inaccessible due to the filter and interdigitiation with neighboring cells, it is possible that many pIgR molecules that reach the basolateral surface are endocytosed before they bind dIgA. Some of these molecules might recycle many times, then eventually bind dIgA, and thereby be stimulated to transcytose. Thus, receptors that are either unoccupied or occupied only slowly could lead to an underestimate of the stimulatory effects of dIgA.

(*ii*) It is possible that ligands dissociate at the apical surface (5, 13, 16) and/or intracellularly. Therefore, the release of ligands into the apical medium may be faster than the cleavage of SC. It is unlikely that intracellular dissociation is a major process: If ligand dissociated in endosomes, we would expect a substantial fraction of the now fluid-phase ligand to be degraded in late endosomes and lysosomes. However, in all cases <5% of ligands were degraded to trichloroacetic acid-soluble products. Also we have found that dissociation of ^{125}I-dIgA is inhibited at the mildly acidic pH found in endosomes (13).

The stimulation of transcytosis by dIgA was probably not due to an increased rate of internalization from the cell surface. Internalization of ^{125}I-Fab and ^{125}I-dIgA both have comparable half-times of ≈1 min with wild-type and mutant pIgRs (16, 17).

Table 1. dIgA and leupeptin cause accumulation of pIgR at the apical surface

Treatment	Biotin pIgR at apical surface*
− dIgA	17.3 ± 2.6
+ dIgA	73.6 ± 1.7

Cells expressing pIgR-Ala^{664} were cultured on 24-mm Transwells. Cells were pretreated with leupeptin at 0.1 mg/ml for 2 hr, which was also present during subsequent manipulations. The cells were labeled with [^{35}S]cysteine for 15 min and chased for 1 hr; where indicated, dIgA at 0.3 mg/ml was present during the chase. After the chase the cells were washed three times with Hanks' buffer at 4°C. The apical surface was incubated with sulfosuccinimidyl-6-(biotinamido)hexanoate at 0.2 mg/ml for two 15-min periods at 4°C, while the basal surface was in contact with MEM/BSA. Immunoprecipitation and analysis were done as in the assay of transcytosis of biotinylated pIgR.

*Results are in arbitrary units generated by the PhosphorImager, expressed as mean ± SEM (n = 3).

Upon reaching the apical surface, the pIgR is proteolytically cleaved to SC. To investigate whether the stimulation of SC release by dIgA is from increased transcytosis or increased cleavage, we pretreated cells expressing pIgR-Ala^{664} with leupeptin, which inhibits cleavage of pIgR to SC (16). Cells were labeled with a pulse of [^{35}S]cysteine and then chased with or without dIgA. Accumulation of uncleaved pIgR at the apical surface was measured by labeling this pIgR with biotin. Table 1 shows that with dIgA there was a 4.2-fold increase in uncleaved pIgR at the apical surface, indicating that dIgA stimulates transcytosis of the pIgR and not just its cleavage.

In all the above experiments we found that similar effects could be obtained with several preparations of human dIgA (including both monoclonal and polyclonal dIgAs). Therefore, our results are not due to the unique properties of one particular preparation of dIgA.

Conceivably the stimulation of transcytosis is not due to specific binding of dIgA to pIgR. However, several negative controls make this hypothesis unlikely. (*i*) Monomeric IgA and pentameric IgM do not bind the rabbit pIgR and did not stimulate transcytosis (Table 2). This result suggests that specific binding to pIgR is necessary. (*ii*) Normally 5% of basolaterally endocytosed ^{125}I-labeled canine transferrin (^{125}I-Tf) is transcytosed to the apical medium (Table 3, ref. 18). Addition of dIgA at 0.3 mg/ml to the basolateral medium did not alter Tf transcytosis, indicating that the stimulation of transcytosis is specific for the pIgR. (*iii*) When ^{125}I-dIgA is used, transcytosis is rapid, yet the total concentration of dIgA is 10 μg/ml. Adding this low concentration of unlabeled dIgA gave no detectable stimulation of transcytosis in the other assays. Finally, we had shown (11) that dIgA is not transcytosed by nontransfected MDCK cells, indicating that transcytosis of dIgA is mediated by the pIgR.

Table 2. Neither monomeric IgA nor pentameric IgM stimulates SC release

Immunoglobulin	SC released apically, %
None	16.8 ± 1.7
Monomeric IgA (0.3 mg/ml)	15.9 ± 0.5
Pentameric IgM (0.3 mg/ml)	17.0 ± 0.3

The pulse-chase assay was done on cells expressing pIgR-Ala^{664} as for Fig. 1 with a 2-hr chase period. The basolateral medium during the chase contained no added immunoglobulin, monomeric IgA, or pentameric human IgM (n = 3).

Table 3. Transcytosis of Tf is not affected by dIgA

Immunoglobulin	^{125}I-Tf transcytosed, %
None	5.5 ± 0.6
dIgA (0.3 mg/ml)	5.0 ± 0.4

Tf (Sigma) was loaded with Fe and iodinated as described (18). Cells internalized the ^{125}I-Tf from the basolateral surface for 1 hr at 37°C. Cells were then washed seven times at 4°C with phosphate-buffered saline. The release of ^{125}I-Tf into the apical medium (transcytosis) over a 1.5-hr chase period was then measured and expressed as a percentage of total ^{125}I-Tf initially internalized (18) (n = 3).

DISCUSSION

Previously we reported (5) that transcytosis is controlled by phosphorylation of Ser-664. Now we report that transcytosis is also stimulated by binding of dIgA. Either dIgA binding or Ser-664 phosphorylation can stimulate transcytosis in the absence or presence of the other factor. Consequently, maximal transcytosis was observed only when both signals were present—i.e., when pIgR-WT was used in the presence of dIgA.

Hirt *et al.* (19) have recently reported that transcytosis of pIgR-Ala^{664} is stimulated by dIgA, which is consistent with our data. They did not investigate the effect of dIgA on transcytosis of pIgR-WT and interpret their data as dIgA rescuing the defect caused by this mutation. Now we find that the stimulation of transcytosis by dIgA is not limited to the pIgR-Ala^{664}. Our data indicate that dIgA stimulates transcytosis of both pIgR-WT and pIgR-Ala^{664}. The stimulation of transcytosis by dIgA was greater when pIgR-Ala^{664} was used. This result may be because the baseline of transcytosis of this mutant in the absence of dIgA is particularly low.

We have recently shown that phosphorylation of Ser-664 is an autonomous signal for transcytosis (20). When the cytoplasmic domain of the pIgR was fused to the extracellular domain of a heterologous reporter protein, mutation of Ser-664 → Ala inhibited transcytosis of the chimera, whereas mutation to aspartate promoted transcytosis. Although stimulation of transcytosis by dIgA binding does not require phosphorylation of Ser-664, dIgA stimulation may involve phosphorylation or other modification of another residue in pIgR (and/or another protein). It is also possible that dIgA binding causes pIgR to dimerize (19), cluster, bind to another protein, or undergo a conformational change. Any of these possibilities could act as a signal that causes the cellular sorting machinery to increase the transcytosis of pIgR. In addition, we cannot exclude the possibility that dIgA selectively binds to a subpopulation of pIgR that has a propensity to be more rapidly transcytosed.

What are the possible physiological roles of stimulation of transcytosis by dIgA binding and phosphorylation? The dIgA must be transported across the epithelial monolayer to protect the organism against pathogens and antigens. The amount of dIgA presented to the basolateral surface of the cell may vary with the tissue and other factors—e.g., local immune responses. Stimulation by ligand could ensure that all bound dIgA is transcytosed, thus adjusting transcytosis to various loads of dIgA.

Phosphorylation presumably controls the transcytosis of the nonligand-bound pIgR and, thus, the level of free SC (i.e., not bound to dIgA) in secretions. In many secretions there is a large excess of free SC (7). Transport of empty pIgR and production of free SC would require that the receptor be phosphorylated, as pIgR without phosphorylation or bound dIgA exhibits very little transcytosis, at least in the MDCK cell system. This result strongly suggests that *in vivo* there is substantial transcytosis of empty pIgR, apparently controlled by phosphorylation of Ser-664. Why does the organism transport empty pIgR and create free SC? SC protects dIgA from proteolytic degradation (7). Presumably a higher concentration of free SC drives the binding reaction so that more dIgA is bound to SC and thereby protected. Phosphorylation could provide a means for the level of free SC to be regulated. Phosphorylation may, in turn, be controlled by an external stimulus—e.g., a cytokine. This result would be analogous to the control of expression of pIgR mRNA by several cytokines (21, 22).

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