Postendocytotic Sorting of the Ligand for the Polymeric Immunoglobulin Receptor in Madin–Darby Canine Kidney Cells

Philip P. Breitfeld,*‡ Jeanne M. Harris,*§ and Keith E. Mostov*

*Whitehead Institute for Biomedical Research, Cambridge, Massachusetts 02142; ‡University of Massachusetts Medical School, Department of Pediatrics, Worcester, Massachusetts 01655; and §Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143

Abstract. The polymeric immunoglobulin receptor (pIg-R) is responsible for the receptor-mediated transcytosis of polymeric immunoglobulins (IgA and IgM) across various epithelia. We have expressed the cDNA for the pIg-R in Madin-Darby canine kidney (MDCK) cells and found that this system mimics that found in vivo (Mostov, K. E., and D. L. Deitcher. 1986. *Cell.* 46:613–621). We have now investigated the postendocytotic pathway of the ligand for the pIg-R. After a 5-min internalization at the basolateral surface, ~45% of internalized ligand recycles to the basolateral medium and 30% is transcytosed to the apical medium.

We have also examined why transcytosis of ligand is unidirectional, going only from basolateral to apical, but not from apical to basolateral. Several factors could explain this, such as proteolytic cleavage of the pIg-R at the apical surface, decreased apical endocytosis of ligand, or an intracellular sorting event. In this report, we show that the protease inhibitor, leupeptin, inhibits the cleavage of the pIg-R but does not alter the unidirectionality of transcytosis. In addition, we demonstrate that there is a significant amount of apical endocytosis of ligand (70% of that observed basolaterally).

Finally, we demonstrate that apically endocytosed ligand can return only to the apical surface. Thus, once ligand reaches the apical surface, it is "trapped" and cannot return to the basolateral surface. We propose that the unidirectionality of transcytosis is the result of intracellular sorting, and that this results from a signal(s) present on the pIg-R.

POLYMERIC immunoglobulins (IgA and IgM) are transported across a variety of epithelia into external secretions. This process of transcytosis is mediated by a receptor that is synthesized and expressed at the cell surface of these transporting epithelia (Mostov and Simister, 1985). This polymeric immunoglobulin receptor (pIg-R)[1] is targeted to the basolateral surface of epithelial cells after synthesis in the rough endoplasmic reticulum and processing in the Golgi stacks. At the basolateral surface, polymeric immunoglobulins bind to the pIg-R and are internalized via receptor-mediated endocytosis. Receptor and ligand are then transported to the apical surface where the extracellular portion of the receptor is cleaved and released along with its ligand (Brandtzaeg, 1974; Mostov and Blobel, 1982; Solari and Kraehenbuhl, 1984). The extracellular portion of the receptor thus formed is termed secretory component (SC).

The biosynthesis and subsequent receptor-mediated transcytosis of pIgR serves as an excellent model system for the study of protein traffic in polarized epithelial cells. We have

characterized the pIg-R in a cell culture system by using a retroviral expression vector to express the pIg-R cDNA in Madin–Darby canine kidney (MDCK) cells (Mostov and Deitcher, 1986). MDCK cells are an epithelial cell line that does not normally express the pIg-R. MDCK cells that express the pIg-R can be cultured on suspended filters such that the basolateral and apical domains are biochemically separate. We have shown that the biosynthetic pathway and function of the pIg-R expressed in MDCK cells cultured on suspended filters mimics that found in vivo (Mostov and Deitcher, 1986).

We have now biochemically examined the pathway of ligand after internalization at the basolateral surface. The pathway of the pIg-R and its ligand has been studied morphologically in rat liver (Takahasi et al., 1982; Geuze et al., 1984). The pIg-R as well as the asialoglycoprotein receptor and the mannose-6-phosphate receptor are all endocytosed at the basolateral surface and enter a morphologically complex endosomal compartment. The pIg-R is segregated away from the other receptors in tubular portions of endosomes, and then is transferred via vesicles to the apical (bile canalicular) surface. To examine the postendocytotic pathway, we have used the MDCK cell system and developed an assay that biochemically follows the fate of a single cohort of baso-

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Dr. Mostov's present address is the Department of Anatomy, University of California, San Francisco, California 94143.

1. *Abbreviations used in this paper:* pIg-R, polymeric immunoglobulin receptor; SC, secretory component.

laterally internalized ligand. We have determined that a substantial portion (~45%) of basolaterally internalized ligand recycles back to the basolateral medium, while 30% is transcytosed and delivered to the apical medium.

A notable feature of this system is that ligand is transcytosed only from the basolateral to the apical surface, and not from the apical to the basolateral surface. Several models could explain this unidirectionality. In one model, after basolateral internalization, the receptor and ligand are randomly delivered to both the basolateral and apical surfaces. Unidirectionality could be achieved if release of ligand occurred preferentially at the apical surface. Events at the cell surface rather than an intracellular sorting event would therefore determine the unidirectionality of transcytosis. Confining the protease that cleaves pIg-R to SC to the apical surface could provide for such a mechanism. Receptors that reach the apical surface could be rapidly cleaved to SC by the protease, which may be confined to this surface. Receptors reaching the basolateral surface could be reendocytosed, and thus reach the apical surface, in further rounds. Thus, the "driving force" for unidirectional transport from basolateral to apical would be receptor cleavage at the apical surface, which renders the transport irreversible. The pIg-R need not contain a signal(s) that directs transport from basolateral to apical. This model assumes that the cleaving protease is exclusively located at or near the apical surface. Previous work suggests that this is very likely. Musil and Baenziger (1987) have shown that receptor cleavage occurs at the cell surface of cultured rat hepatocytes. In addition, we have shown that a mutant pIg-R lacking the entire cytoplasmic domain is targetted to the apical surface only and yet is cleaved to SC (Mostov et al., 1986). Finally, Musil and Baenziger (1988) have isolated bile canalicular (apical) membranes from rat hepatocytes and have shown in vitro that these membranes contain a proteolytic activity capable of cleaving pIg-R into SC.

Cell surface events other than SC cleavage may determine the unidirectionality of transcytosis. Transcytosis could be rendered unidirectional by markedly slow or decreased endocytosis of ligand at the apical surface. Ligand reaching the apical surface would therefore be less likely to return to the basolateral surface. Alternatively, ligand may be preferentially released into the apical medium if it is unable to be released into the basolateral medium since the receptor is not cleaved to SC at the basolateral surface, and if the ligand is unable to dissociate from receptor at the basolateral surface. Finally, the unidirectionality could be achieved as a result of intracellular, postendocytotic sorting. In this model, the pIg-R would contain a (sorting) signal which directs it from the endosome to the apical surface.

In this report, we have tested these various models and conclude that the final model is most likely. We propose that the polarity and unidirectionality of transcytosis is a result of intracellular sorting, most likely determined by a sorting signal contained within the pIg-R.

Materials and Methods

Growth of MDCK Cells on Suspended Filters

MDCK cells were cultured on either surfactant-free 0.45-μm pore size chambers (Millicell HA; Millipore Continental Water Systems, Bedford,

MA) or 0.4-μm pore size chambers (transwell; Costar, Cambridge, MA). These units are placed in a 24-well tissue culture plate (Corning Glass Works, Corning, NY) and prewet with medium for 5-min at room temperature. Cells from a confluent 10-cm tissue culture dish were removed with trypsin, washed with medium, and resuspended in 10 ml of medium. An aliquot of cells (0.4 ml for millicell HA chambers and 0.2 ml for transwell chambers) was added to each chamber. Fresh medium was added daily. Chambers were used for study after 3 or 4 d of growth. Cells were grown in MEM with 10% FCS, penicillin, and streptomycin. Parent MDCK cells or MDCK cells that express the cDNA for the pIg-R were used for study and have been described previously (Mostov and Deitcher, 1986; Breitfeld et al., in press).

Guinea Pig Anti-SC Fab Fragments

SC was purified from rabbit bile (Pel-Freez Biologicals, Rogers, AR). PMSF was added to 1 mM, and the bile was dialyzed against 3 12-h changes of 100 vol 0.15 M NaCl. About 1 ml of bile was loaded onto each of 6 preparative SDS 7% polyacrylamide gels using the Laemlli system without reducing agent. After staining with Coomassie blue, the SC band was excised, lyophilized, and ground with a mortar and pestle. Antibodies were raised in guinea pigs (Ribi ImmunoChem Research, Inc., Hamilton, MI) by subcutaneously injecting 50 mg of ground gel every 3 wk for 6 injections, using the adjuvant supplied by Ribi ImmunoChem Research, Inc. Animals were bled 7–10 d after each of the last 3 injections. Specificity of the guinea pig antiserum for the pIg-R was determined by its ability to immunoprecipitate the pIg-R (data not shown) and by its specific binding only to cells that express the pIg-R (see Table I). In addition, binding of [^{125}I]anti-SC Fab fragments to pIg-R can be competed in a concentration-dependent manner by addition of unlabeled anti-SC Fab fragments. At 100× excess of unlabeled ligand, binding of labeled ligand is reduced by 93%.

Affinity-purified antibody was isolated by preparing an affinity resin. Dialyzed bile (20 ml) was made to 2% SDS and boiled for 10 min. This was then coupled to 10 ml of CNBr-activated Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) using the manufacturer's directions. Two ml of antiserum were diluted with 20 ml of buffer (1% Triton-X-100, 0.2% SDS, 150 mM NaCl, 5 mM EDTA, 8% sucrose, 0.1% NaN$_3$, 10 U/ml trasylol, and 20 mM triethandamine-HCl, pH 8.6), applied to the affinity column, and recirculated overnight at 4°C. The column was then washed with 3-column vol of the above buffer, followed by 2-column vol washes with buffer lacking detergents and finally 2-column vol of H$_2$O. Elution was with 5 vol of 1% triethylamine in H$_2$O (pH not adjusted). The eluate was neutralized with 1 M Hepes-NaOH, pH 7.4, and concentrated 10-fold with a concentrator (Centricon; Amicon Corp., Danvers, MA). Fab fragments were generated using a kit from Pierce Chemical Co. (Rockford, IL).

Iodination of Proteins

Both human dIgA (the gift of Drs. J. M. Schiff and B. Underdown, University of Toronto) and anti-SC Fab fragments were iodinated by the iodine monochloride method (Goldstein et al., 1983) to a specific activity of 1 × 10^7 cpm/μg for dIgA and 5 × 10^7 cpm/μg for anti-SC Fab fragments. Unincorporated ^{125}I was removed by dialysis.

Binding, Transcytosis, and Internalization Assays

For these experiments, cells were manipulated in medium consisting of MEM plus 0.6% BSA and 20 mM Hepes, pH 7.3. For binding experiments, cells grown on suspended filters were rapidly cooled by placing them in precooled medium at 4°C in a 4°C room, and were allowed to bind either [^{125}I]dIgA (at 1 μg/ml) or [^{125}I]anti-SC Fab fragments (at 4 μg/ml). Binding was performed for 2 h at 4°C. To remove nonspecifically bound molecules, cells were washed extensively with medium at 4°C for 60 min. This washing removes essentially all nonspecifically bound Fab fragments, since MDCK cells that do not express the pIg-R have virtually no Fab binding compared to cells that express the pIg-R (see Table I).

For internalization experiments, cells were rapidly warmed to 37°C for 5 min to allow for internalization and then rapidly cooled to 4°C (as described above). To remove cell surface bound but noninternalized ligand, the basolateral surface of cells was incubated with a mixture of 50 μg/ml chymotrypsin and 50 μg/ml proteinase K in PBS, containing calcium and magnesium, for 60 min in two successive 30-min incubations, at 4°C. Cells were washed for 15 min with medium containing 10% FCS, and rewarmed to 37°C. Medium from the apical or basolateral chamber was harvested at the appropriate time and made to 15% trichloroacetic acid (TCA). Samples

Table I. Binding of Ligand to MDCK Cell Surface

Ligand	Cell line	Apical (pg/filter)	Basolateral (pg/filter)
Anti–SC Fab	+pIg-R	592	508
	−pIg-R	24	12
dIgA	+pIg-R	4,063	845
	−pIg-R	246	434

[^{125}I]Anti–SC Fab fragments (2 μg/ml) or [^{125}I]dIgA (4 μg/ml) were bound at 4°C for 2 h to either the apical or basolateral surface of either MDCK cells expressing the pIg-R (+pIg-R) or the parent MDCK cell line (−pIg-R). Cells were extensively washed for 60 min, and the amount of cell-associated radioactivity was determined in a gamma counter.

were precipitated in TCA for 1 h at 4°C, and TCA soluble (degraded) and insoluble (intact) material was separated by centrifugation at 15,000 g for 15 min at 4°C. Samples were quantitated in a gamma counter (Packard Instrument Co., Inc., Downers Grove, IL). Unless otherwise indicated, all figures represent TCA insoluble (intact) radioactivity (degraded radioactivity represented <5% of intact radioactivity in all cases).

For transcytosis experiments involving a single cohort of basolaterally bound molecules, cells were cooled to 4°C and allowed to bind ligand as described above. After extensive washing, cells were rapidly warmed to 37°C and the apical medium was sampled at various times. Harvested medium was precipitated with TCA as described above.

For continuous uptake and transcytosis experiments, radio-iodinated ligand (dIgA at 0.2 μg/ml or anti–SC Fab fragments at 0.8 μg/ml) was added to the basolateral medium at 37°C. Cells were then incubated at 37°C for various periods of time. The apical medium was sampled, precipitated with TCA and the amount of radioactivity was determined as above.

Metabolic Labeling and Analysis

Cells grown on filters were washed twice with PBS at 37°C and then incubated with MEM lacking cysteine and containing 5% dialyzed FCS for 15 min at 37°C. After this period of cysteine starvation, each filter was placed directly onto a 10-μl drop of MEM lacking cysteine but containing 5% dialyzed FCS and 30 μCi[^{35}S]cysteine (New England Nuclear, Boston, MA). The cells were incubated at 37°C for the designated pulse period. At the end of the pulse period, the filters were transferred to prewarmed 24-well tissue culture plates with 200 and 400 μl of MEM with 10% FCS 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 goat anti–rabbit SC antibody as previously described (Mostov and Deitcher, 1986). Immunoprecipitates were analyzed by SDS 7% PAGE and fluorography.

Results

The postendocytotic pathway of internalized pIg-R and its ligand has been morphologically examined in rat liver (Takahasi et al., 1982; Geuze et al., 1984). Since we have expressed the pIg-R in polarized MDCK cells and are able to culture them on suspended filters, this system has the potential to allow us to follow this pathway biochemically. Up to this point, there have been two factors that have limited our ability to develop such an approach. First, radio-iodinated dIgA, when used for basolateral binding assays, produces an unacceptable level of background binding as a consequence of nonspecific binding to the filter on which cells are cultured. Thus, a ligand with less nonspecific binding needed to be developed. Second, a method to isolate a single cohort of internalized ligand had to be developed. This method would have to incorporate a procedure to strip efficiently the basolateral surface of bound, noninternalized ligand, and yet not disrupt the MDCK cell monolayer.

Anti–SC Fab Fragments Function as Ligand

We sought to develop a ligand that would function as native

ligand (dIgA) yet display a low amount of nonspecific binding at the basolateral surface of MDCK cells that express the pIg-R. To address this issue, Fab fragments from affinity-purified guinea pig anti–SC antiserum were generated. Steady-state ligand binding as well as functional assays were performed to compare anti–SC Fab fragments to dIgA.

MDCK cells expressing the pIg-R and parent MDCK cells were cultured on suspended filters and allowed to bind [^{125}I]dIgA or [^{125}I]anti–SC Fab fragments at 4°C for 2 h and then extensively washed. Binding was performed at the apical or the basolateral surface. The results are displayed in Table I. At the apical surface, both anti–SC Fab fragments and dIgA yielded a specific to nonspecific binding ratio of 20–40:1 (nonspecific binding is defined as the amount of binding observed for parent MDCK cells). Thus, at steady state, the apical surface displays a substantial amount of ligand binding, apparently because of intact pIg-R. Since cleavage of the pIg-R to SC occurs at the apical surface (Musil and Baenziger, 1988), why do we observe intact pIg-R at the apical surface? A likely explanation is that once pIg-R reaches the apical surface, it is not instantaneously cleaved to SC. Thus, a pool of uncleaved pIg-R is present at the apical surface. We have previously shown this to be the case in nonpolarized fibroblasts, where surface iodinated pIg-R is cleaved to SC with a half-time of ∼5–10 min (Deitcher et al., 1986). We have also demonstrated the presence of intact pIg-R at the apical surface of MDCK cells by radio-iodination of the apical surface by the lactoperoxidase-glucose oxidase method (Hubbard and Cohn, 1975; Breitfeld et al., unpublished observations).

When dIgA and anti–SC Fab fragment binding is compared at the basolateral surface, a specific to nonspecific binding ratio of greater than 40:1 was obtained with anti–SC Fab fragments whereas dIgA yielded a ratio of 2:1 (see Table I). Thus, anti–SC Fab fragments demonstrated substantially less background binding at the basolateral surface than native ligand (dIgA).

To determine if anti–SC Fab fragments would be transported across the MDCK cell monolayer from the basolateral to apical medium, as is native ligand, radio-iodinated anti–SC Fab fragments or dIgA was placed in the basolateral medium and cells were incubated at 37°C for various periods of time up to 18 h. The apical medium was assayed for the amount of ligand transported. Fig. 1 A demonstrates that anti–SC Fab fragments are specifically transported from the basolateral to the apical medium in a fashion similar to native ligand (dIgA). We regularly observe more transcytosis of anti–SC Fab fragments on a weight basis as compared to dIgA, most likely the result of using a higher concentration of anti–SC Fab fragments over dIgA. Another possibility is that the Fab fragments (derived from a polyclonal antiserum) have multiple binding sites on the pIg-R whereas there may be only one binding site per receptor for dIgA. The dIgA may also lose some of its biological activity as a result of radio-iodination. There is very little nonspecific transport of either anti–SC Fab fragments of dIgA since the parent cell line (MDCK cells) transports <5% of that transported by cells that express the pIg-R (Fig. 1 A). Transport from the apical to basolateral medium is less than 5% of that in the opposite direction and does not differ from the parent MDCK cell line.

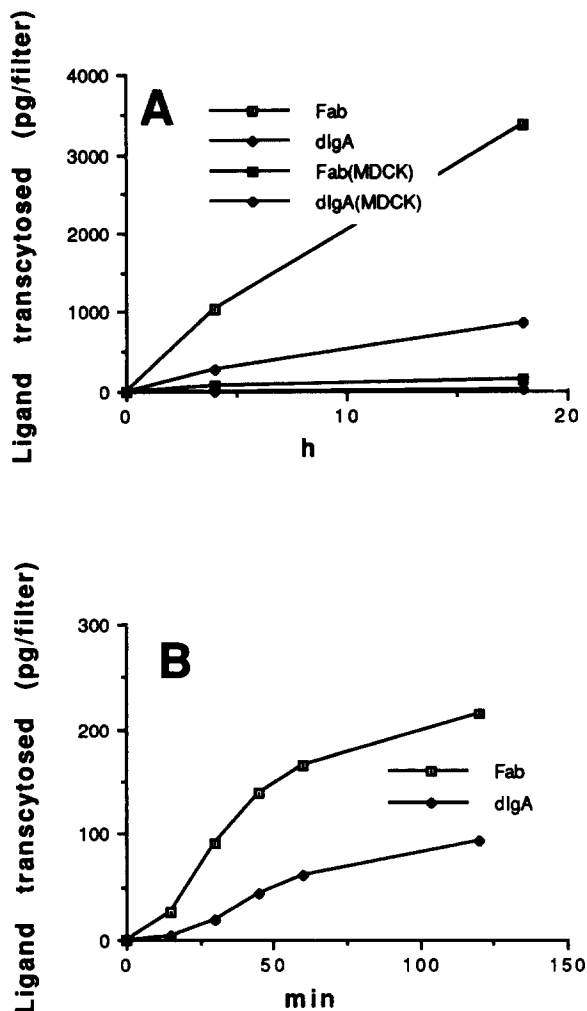The transcytosis of a single cohort of bound anti–SC Fab

Figure 1. Continuous (A) or single round (B) transcytosis of dIgA and anti–SC Fab fragments. (A) Continuous transcytosis. ^{125}I-Labeled dIgA (0.2 µg/ml) or anti–SC Fab fragments (0.8 µg/ml) were added to the basolateral medium and the cells (either MDCK cells expressing pIg-R or parent MDCK cells) were incubated at 37°C. At 4 and 18 h, the apical medium was collected and TCA precipitated, and the insoluble material was counted in a gamma counter. Points were performed in duplicate and varied by <10%. Fab (MDCK) and dIgA (MDCK) refer to the amount of transcytosis of Fab fragments or dIgA, respectively, observed for the parent MDCK cells. (B) Single round transcytosis. ^{125}I-Labeled dIgA (1 µg/ml) or anti–SC Fab fragments (4 µg/ml) were bound to the basolateral surface for 2 h at 4°C, and the basolateral surface was extensively washed. Cells were rapidly warmed to 37°C, and the apical medium was collected at the times indicated. Medium was TCA precipitated and the insoluble material was counted in a gamma counter. Points were performed in duplicate and varied <10%.

fragments was also examined and compared to dIgA. Anti–SC Fab fragments or dIgA were bound at 4°C to the basolateral surface of MDCK cells expressing the pIg-R and warmed to 37°C. At various times, the apical medium was assayed for appearance of ligand. Fig. 1 B demonstrates that the time course of transcytosis is similar for both anti–SC Fab fragments and dIgA, with the half-time of the process being ~45 min. Again, we observe more transcytosis with anti–SC Fab fragments. Approximately one third of bound ligand is transcytosed during the experiment. TCA soluble

material (degraded ligand) accounted for <5% of bound radioactivity. We routinely TCA precipitated the medium in all subsequent experiments. Transcytosis medium was also analyzed by SDS 7% PAGE and demonstrated that >95% of transcytosed radioactivity is in the form of intact Fab fragments (data not shown). Thus, anti–SC Fab fragments specifically recognize the pIg-R at the cell surface with very little nonspecific binding and they display the ability to undergo transcytosis similar to native ligand. This reagent was used as ligand for subsequent studies. Similarly, Beisiegel et al. (1981) have demonstrated the usefulness of antibodies against receptor proteins as probes, or "pseudoligands," for the study of receptor mediated events.

Fate of Basolaterally Internalized Ligand

To follow the fate of a single cohort of basolaterally internalized ligand, an internalization assay was developed to isolate such a cohort. This required a procedure that would efficiently strip the basolateral surface of cell surface bound, noninternalized ligand and yet not disrupt the MDCK cell monolayer.

Radio-iodinated anti–SC Fab fragments were bound to the basolateral surface of cells at 4°C, washed extensively, and then rapidly warmed to 37°C for 5 min to allow internalization of ligand. Cells were then rapidly cooled to 4°C and exposed to gentle proteolysis at the basolateral surface with 50 µg/ml of both chymotrypsin and proteinase K for 60 min at 4°C. This removes 80–85% of cell-surface bound ligand (Table II).

We were concerned that this proteolysis might damage the monolayer. We therefore examined the effect of proteolysis on the ability of the monolayer to prevent the nonspecific leakage of anti–SC Fab fragments across the monolayer. We used the parent MDCK cells that do not express the pIg-R. These cells were incubated at 4°C without or with basolateral or apical chymotrypsin and proteinase K for 60 min. Cells were then incubated for 1 h at 37°C with radio-iodinated anti–SC Fab fragments added to the side that was exposed to proteases. At the end of the 1-h incubation at 37°C, the medium opposite to the added radioactivity was harvested and TCA precipitated. The TCA precipitated radioactivity represents the amount of intact Fab fragments that leaked across the monolayer during the incubation. Table III demonstrates the results. There was only a slight increase in the amount of intact Fab fragments that leaked across the monolayers pretreated with proteases. In all cases, the amount of nonspecific leakage of anti–SC Fab fragments is

Table II. Effect of Protease Treatment on Surface-bound Ligand

	pg/filter	Percentage of bound (%)
Bound	1,392.9	100.0
Protease-sensitive	1,123.0	80.7
Protease-resistant	269.9	19.3

[^{125}I]Anti–SC Fab fragments were bound to the basolateral surface of MDCK cells at 4°C for 2 h, and then the basolateral surface was washed extensively. The basolateral surface was then exposed to a mixture of 50 µg/ml chymotrypsin and 50 µg/ml proteinase K for 60 min at 4°C. The protease-sensitive fraction is that which is removed by the protease treatment. The protease-resistant fraction is that which remains cell-associated after protease treatment. The amount bound is the sum of the two fractions.

Table III. Effect of Proteolysis on MDCK Monolayer

Ligand	Proteases	Transport across monolayer (pg/filter)
Apical	None	0.24
Apical	Apical	0.32
Basal	None	0.16
Basal	Basal	0.24

Parent MDCK cells were preincubated at 4°C with or without proteinase K and chymotrypsin at 50 μg/ml each in two successive 30-min incubations. After washing with medium for 15 min, cells were warmed to 37°C and [^{125}I]anti-SC Fab fragments (2 μg/ml) were added to the appropriate medium. After 1 h, the medium opposite the added Fab fragments was harvested and TCA precipitated. The amount of radioactivity was determined in a gamma counter.

much less than the transcytosis observed with cells that express the pIg-R (see Fig. 1 A). Similar proteolysis protocols have been used by others to examine the endocytosis of Semliki Forest virus (Marsh et al., 1983), the surface delivery of hemagglutinin in MDCK cells (Matlin and Simons, 1984), and the movement and function of the asialoglycoprotein receptor (Breitfeld et al., 1984), and in these cases, no effects of proteolysis on membrane traffic were observed. Thus, gentle proteolysis does not substantially alter the MDCK monolayer. However, we cannot rule out the possibility that some cellular events may be affected.

This treatment efficiently strips the basolateral surface of ligand so that a single cohort of basolaterally internalized ligand is created. The fate of this cohort can then be followed biochemically. After a 5-min incubation at 37°C, 80% of bound anti-SC Fab fragments become resistant to proteolysis, presumably because of internalization at the basolateral surface. After proteolysis at the basolateral surface, we therefore rapidly rewarmed the cells to 37°C. At various times, both the basolateral and apical medium is sampled for the appearance of ligand. Each sample was TCA precipitated.

Fig. 2 demonstrates that ∼30% of ligand internalized over 5 min is released into the apical medium and that the half-time of this process is ∼15–20 minutes. A significant fraction (45%) of internalized ligand rapidly reappears intact (TCA-precipitable) in the basolateral medium with a half time of ∼6 min. This finding is particularly significant since it has been assumed by many investigators, including ourselves, that once the pIg-R had internalized ligand, the ligand had only one destination, the apical medium. Only 5% of internalized ligand was released degraded (TCA soluble) and 20% was still cell-associated at the end of the 90-min incubation. We do not know why 20% of internalized ligand remains cell associated. A similar phenomenon has been observed in rat liver (Hoppe et al., 1985).

What is the mechanism of ligand recycling? It cannot be because of basolateral cleavage of receptor to SC since <5% of SC recovered in the medium is found basolaterally (see Fig. 3, lanes 6, 8, and 10). Two explanations seem plausible. First, receptor and ligand may recycle together, both returning to the basolateral surface. There, some ligand may dissociate into the medium while an unknown amount may remain receptor-bound and undergo a second round of internalization. If this model is correct, our measurements will underestimate the true amount of ligand returning to the basolateral surface. Second, before return to the basolateral

surface, ligand may dissociate from receptor intracellularly. Ligand could then recycle by a fluid phase process. No matter which model we choose, dissociation of ligand from receptor is apparently occurring, either at the basolateral surface or intracellularly. Also, these models reveal important limitations on interpreting our data. Specifically, our data refers to ligand movement, and we cannot directly infer the pathway of the receptor from this data.

With our ability to biochemically monitor the postendocytotic pathway of the ligand for the pIg-R, we asked whether or not the cleavage of the receptor played a role in directing ligand to either final destination. The hypothesis that the proteolytic cleavage of the pIg-R, with subsequent release of SC and bound dIgA, drives the movement of ligand to the apical surface is attractive especially if one assumes that the proteolytic cleavage occurs at or near the apical surface. We sought to directly test this hypothesis by inhibiting proteolytic cleavage of the receptor with leupeptin and determining if this inhibition has an effect on the transcytosis of ligand.

Effect of Leupeptin on pIg-R Biosynthesis in MDCK Cells

Leupeptin is an inhibitor of thiol proteases (Bond and Butler, 1987). Musil and Baenziger (1987) have shown that leupeptin inhibits the cleavage of the pIg-R, and therefore release of SC, in rat hepatocyte monolayers. Therefore, we examined the effect of leupeptin on MDCK cells that express the pIg-R by assessing the biosynthesis and turnover of the pIg-R in the absence and presence of leupeptin.

Cells were starved with MEM without cysteine and then metabolically labeled with [^{35}S]cysteine for 20 min and chased for various periods of time up to 8 h in the absence or presence of 0.1 mg/ml leupeptin. At the end of the chase period,
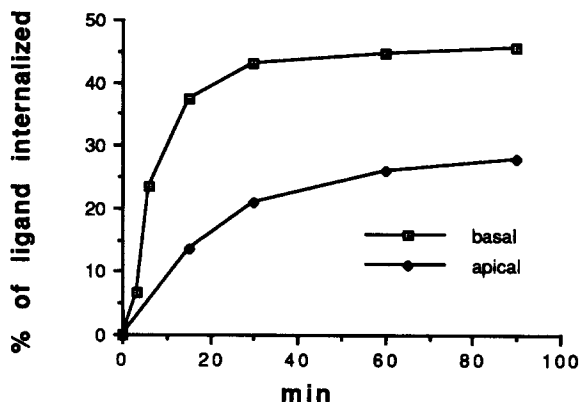
Figure 2. Fate of basolaterally internalized ligand. ^{125}I-Labeled anti-SC Fab fragments (4 μg/ml) were bound to the basolateral surface for 2 h at 4°C, and the basolateral surface was extensively washed. The cells were rapidly warmed to 37°C for 5 min and rapidly cooled to 4°C. The basolateral surface was exposed to a mixture of 50 μg/ml chymotrypsin and 50 μg/ml proteinase K for 60 min at 4°C. After washing, cells were rewarmed to 37°C, and the apical and basolateral medium was collected at the times indicated. Samples were TCA precipitated and the insoluble material was counted in a gamma counter. Points were performed in duplicate and varied <5%. Values are expressed as a percentage of the total amount of ligand internalized during the initial 5-min incubation at 37°C.
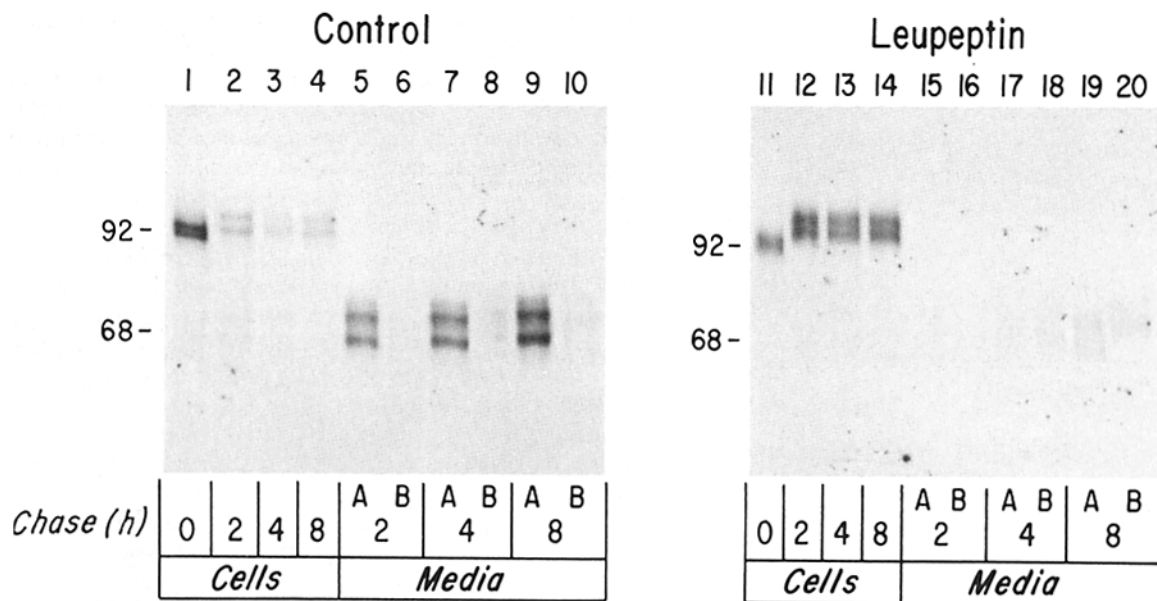
Figure 3. Effect of leupeptin on biosynthesis and processing of the pIg-R. Cells cultured on suspended filters were metabolically labeled with [³⁵S]cysteine for 15 min and chased for the times indicated, in the absence (*Control*) or presence (*Leupeptin*) of leupeptin at 0.1 mg/ml. Cells as well as apical and basolateral media samples were harvested and immunoprecipitated with anti-SC antibody and protein A-Sepharose. The products were analyzed by 7% SDS-PAGE and fluorography. *A* indicates apical medium and *B* indicates basolateral medium. Molecular mass markers are indicated at the left (phosphorylase A 92 kD; BSA 68 kD).

cells and media were immunoprecipitated with anti-SC antiserum and the products were analyzed by SDS-PAGE and fluorography. As previously reported, Fig. 3 demonstrates that immediately after the pulse period, the pIg-R is identified as a single polypeptide of ∼90 kD (lane *1*) and is converted to a doublet of ∼100 kD and ∼105 kD during the subsequent chase periods (lanes *2–4*). This is the mature form of the pIg-R and has complex oligosaccharides. In addition, by 2 h of chase, a group of products of ∼68 kD appears in the apical medium (lane *5*). This represents cleavage of the pIg-R and release of SC into the apical medium. As the chase period progresses, more SC accumulates in the apical medium with a concomitant decrease in the amount of mature intracellular pIg-R.

When cells are labeled and chased in the presence of 0.1 mg/ml leupeptin, a single polypeptide species of ∼90 kD is identified (Fig. 3, lane *11*), and this species is converted to the mature form of the pIg-R by 2 h of chase (lane *12*), as was observed similarly with control cells. However, almost no SC is recoverable in the apical medium during the entire chase period (lanes *15*, *17*, and *19*). Thus, in the presence of leupeptin, the initial synthesis and Golgi body processing of the pIg-R proceeds normally. Yet the usual mode of degradation of the pIg-R is greatly affected by leupeptin since very little SC is released into the apical medium. In addition, leupeptin, presumably by inhibiting SC cleavage, leads to a marked prolongation of pIg-R half-life from roughly 2 h to >8 h.

By metabolically labeling cells with [³⁵S]cysteine for 30 min and chasing for 4 h in the absence or presence of leupeptin in concentrations up to 1 mg/ml, we found that the inhibition of SC cleavage by leupeptin was dose dependent. The amount of SC released into the apical and basolateral medium was quantitated by densitometry of the immunoprecipitated products. The inhibition was 85% complete at 0.1 mg/ml

leupeptin. We used 0.1 mg/ml leupeptin for our subsequent studies.

Effect of Leupeptin on Transcytosis

Since leupeptin prevents cleavage of the pIg-R, one might expect that the release not only of SC but also of ligand into the apical medium might be impaired in the presence of leupeptin. Given the ability to directly measure polarized transcytosis in this system, we determined the ability of cells exposed to leupeptin to transport ligand from the basolateral to apical medium.

MDCK cells expressing the pIg-R were pretreated without or with leupeptin for 2 h. Radio-iodinated anti-SC Fab fragments were then added to the basolateral medium, and cells were incubated for 18 h in the absence or presence of leupeptin. The apical medium was sampled at various times for the presence of intact (TCA precipitable) ligand. Fig. 4 *A* demonstrates that cells exposed to leupeptin transcytose anti-SC Fab fragments. At 18 h, cells exposed to leupeptin have transported slightly more anti-SC Fab fragments compared to cells not exposed to leupeptin. Similar results were obtained using dIgA as ligand (data not shown). This transport is specific since cells that do not express the pIg-R (parent MDCK cells) do not significantly transport anti-SC Fab fragments in the presence or absence of leupeptin (Fig. 4 *A*). Transcytosis from the apical to the basolateral medium was <5% of transcytosis from the basolateral to apical medium in the absence or presence of leupeptin, and was no greater than that observed in the parent MDCK cell line (data not shown).

The effect of leupeptin on a single cohort of basolaterally bound ligand was also determined. In this analysis, anti-SC Fab fragments are bound to the basolateral surface of MDCK cells at 4°C. The cells are then rapidly warmed to 37°C without any treatment with proteases, and the appearance of li-
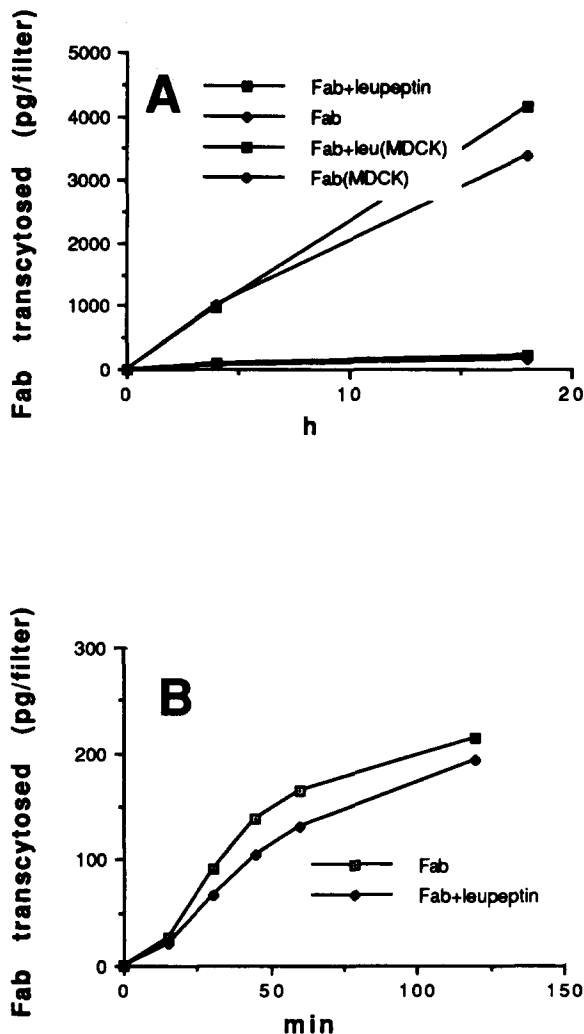
Figure 4. Effect of leupeptin on continuous (*A*) and single round (*B*) transcytosis. (*A*) Continuous transcytosis. ^{125}I-Labeled anti–SC Fab fragments (0.8 μg/ml) were added to the basolateral medium of cells expressing the pIg-R (*open squares and solid triangles*) or parent MDCK cells (*solid squares and open triangles*) and incubated at 37°C in the absence or presence of 0.1 mg/ml leupeptin. At 4 and 18 h, the apical medium was collected and TCA precipitated, and the insoluble material was counted in a gamma counter. Points were performed in duplicate and varied by <10%. (*B*) Single round transcytosis. Cells cultured on suspended filters were treated for 2 h without or with 0.1 mg/ml leupeptin at 37°C. ^{125}I-labeled anti–SC Fab fragments (4 μg/ml) were bound to the basolateral surface for 2 h at 4°C, and the basolateral surface was extensively washed. Cells were rapidly warmed to 37°C and the apical medium was collected at the times indicated. Medium was TCA precipitated, and the insoluble material was counted in a gamma counter. Points were performed in duplicate and varied <10%. The entire experiment was performed in the absence (*open squares*) or presence (*solid triangles*) of 0.1 mg/ml leupeptin.

gand in the apical medium is determined at various times up to 120 min. Fig. 4 *B* demonstrates that in the absence or presence of leupeptin, anti–SC Fab fragments are transported into the apical medium in similar amounts and with similar kinetics.

In summary, despite inhibition of SC release into the apical medium, leupeptin does not impair the continuous uptake and transcytosis of ligand nor the uptake and transcytosis of

a single cohort of basolaterally bound ligand. The absence of any significant apical to basolateral transport of anti–SC Fab fragments in the absence or presence of leupeptin, as well as the lack of an effect of leupeptin on transcytosis, makes it unlikely that the proteolytic cleavage of the pIg-R at the apical surface determines the directionality of transcytosis.

Effect of Leupeptin on the Fate of Basolaterally Internalized Ligand

Since we have demonstrated that basolaterally internalized ligand has two major fates (Fig. 2), we examined the effect that leupeptin might have on the postendocytotic pathway of
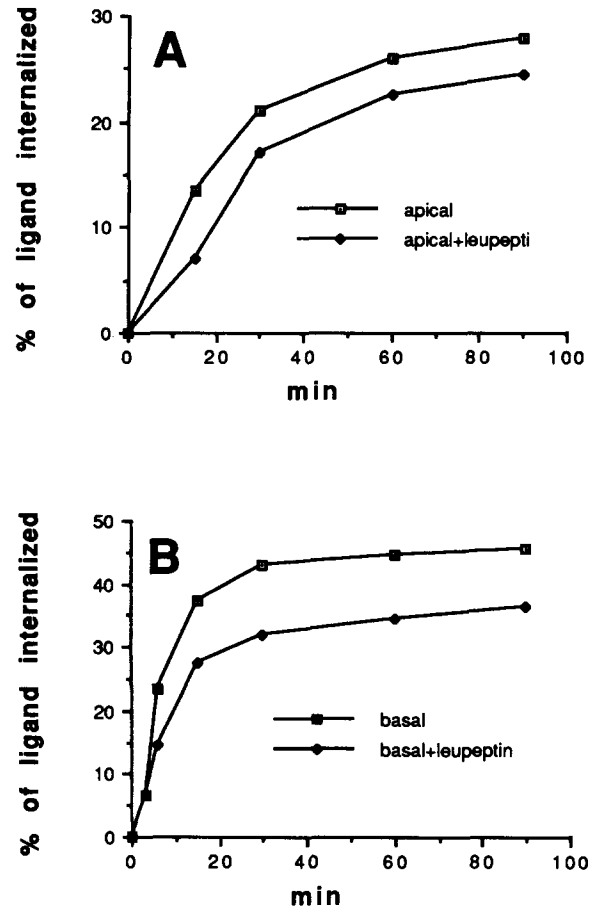

Figure 5. Effect of leupeptin on the fate of basolaterally internalized ligand. Cells cultured on suspended filters were treated for 2 h without or with 0.1 mg/ml leupeptin at 37°C. ^{125}I-Labeled anti–SC Fab fragments (4 μg/ml) were bound to the basolateral surface for 2 h at 4°C, and the basolateral surface was extensively washed. The cells were rapidly warmed to 37°C for 5 min and rapidly cooled to 4°C. The basolateral surface was exposed to a mixture of 50 μg/ml chymotrypsin and 50 μg/ml proteinase K for 60 min at 4°C. After washing, cells were rewarmed to 37°C, and the apical (*A*) and basolateral medium (*B*) was collected at the times indicated. The entire experiment was performed in the absence or presence of 0.1 mg/ml leupeptin. Samples were TCA precipitated and the soluble and the insoluble material was counted in a gamma counter. Points were performed in duplicate and varied <5%. Values are expressed as a percentage of the total amount of ligand internalized during the initial 5-min incubation at 37°C.
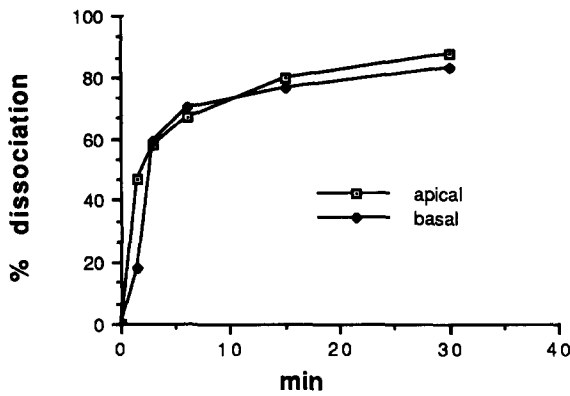
Figure 6. Dissociation of ligand from the apical and basolateral surface at 37°C. Cells expressing the tail-minus mutant pIg-R were incubated for 2 h in the presence of 0.1 mg/ml leupeptin at 37°C. Cells were cooled to 4°C and ^{125}I-labeled anti-SC Fab fragments (4 μg/ml) were bound to the apical or basolateral surface for 2 h. The appropriate surface was extensively washed. The cells were rapidly warmed to 37°C, and the apical or basolateral medium was collected at the times indicated. The entire experiment was performed in the presence of 0.1 mg/ml leupeptin. Samples were TCA precipitated, and the insoluble material was counted in a gamma counter. Points were performed in duplicate and varied by <10%.

the ligand. MDCK cells were preincubated with 0.1 mg/ml leupeptin for 2 h at 37°C and allowed to bind anti-SC Fab fragments at 4°C at the basolateral surface, washed extensively, and then rapidly warmed to 37°C for 5 min to allow basolateral internalization. Cells were then placed on ice and exposed to gentle proteolysis at the basolateral surface (described above) and again warmed to 37°C. At various times the apical and basolateral medium was sampled for appearance of ligand. The entire experiment was performed in either the absence or presence of 0.1 mg/ml leupeptin.

Fig. 5 A demonstrates that in the absence or presence of leupeptin, similar amounts of basolaterally internalized ligand are transcytosed into the apical medium, although transcytosis may be somewhat slower in the presence of leupeptin. That transcytosis occurs in the absence of SC cleavage implies that simple dissociation of ligand from receptor is occurring at or near the apical surface. Fig. 5 B demonstrates that ligand internalized from the basolateral surface also recycles to the basolateral surface in the absence or presence of leupeptin. There is slightly less transcytosis as well as basolateral recycling in the presence of leupeptin. Leupeptin may generally slow membrane flow and account for this mild effect. However, there does not appear to be a significant alteration in the fate of internalized ligand in the presence of leupeptin. We conclude that inhibition of SC cleavage by leupeptin does not substantially alter the postendocytotic pathway of ligand basolaterally internalized by the pIg-R.

Dissociation of Ligand at the Apical and Basolateral Surface

Since we have shown that inhibition of SC cleavage by leupeptin does not impair transcytosis of ligand into the apical medium (Figs. 4 and 5), by what mechanism is ligand released into the apical medium? It is likely that, with leupeptin treatment, ligand simply dissociates from intact receptor at or near the apical surface without the need for SC cleavage.

To determine the rate of dissociation of ligand from intact pIg-R at 37°C, we used a previously described mutant of the pIg-R (tail-minus receptor) (Mostov et al., 1986). This mutant pIg-R lacks the entire cytoplasmic domain and is anchored to the membrane by the membrane spanning region. Importantly, this receptor fails to internalize ligand at the cell surface at 37°C. MDCK cells expressing the tail-minus receptor were pretreated with 0.1 mg/ml leupeptin (to inhibit receptor cleavage) for 2 h. Fab fragments were bound to either the apical or basolateral surface at 4°C for 2 h, and the appropriate surface extensively was washed to remove nonspecific binding. The cells were rapidly warmed to 37°C and the apical or basolateral medium was sampled for the appearance of ligand. The entire experiment was performed in the presence of leupeptin. Using the tail-minus mutant and leupeptin allows the measurement of dissociation of Fab fragments from receptor at 37°C without the confounding variables of receptor internalization and receptor cleavage. The results are displayed in Fig. 6. The rates of dissociation of Fab fragments from receptor at 37°C are remarkably similar at both the apical and basolateral surfaces. Initially, the dissociation at the basolateral surface may be slower than at the apical surface (similar to the observed rates of dissociation at 4°C, data not shown), although this may simply reflect diffusion through the filter on the basolateral side. In any case, this observed spontaneous release of ligand from the cell surface in the presence of leupeptin provides a mechanism for delivery of ligand into the medium without SC cleavage at either the apical or basolateral surfaces.

Why is the binding of the anti-SC Fab fragments so weak that they dissociate this quickly? The anti-SC Fab fragments used in this study have been affinity-purified, and thus the preparation consists of molecules of moderate affinities for the pIg-R, while those with the highest affinity remained on the affinity column. Furthermore, we affinity purified the divalent IgG, but then cleaved them to monovalent Fab. The avidity of the monovalent fragment is expected to be the square root of the divalent IgG; i.e., much weaker. Both of these factors may explain spontaneous dissociation from receptor. In addition, similar dissociation experiments have been performed with dIgA (native ligand) that also dissociates from receptor in the presence of leupeptin at both the apical and basolateral surfaces (data not shown). It should be appreciated that if dissociation at 37°C was not rapid, we could not measure basolateral recycling or transcytosis in the presence of leupeptin.

Apical Binding and Internalization of Anti-SC Fab Fragments

Assuming that molecules internalized by the pIg-R are randomly delivered to the apical and basolateral surfaces, unidirectionality of transcytosis could still be achieved without cleavage of receptor at the apical surface if (a) there were no apical binding of ligand, (b) there was an enhanced rate of dissociation of ligand from receptor at the apical surface compared to the basolateral surface, or (c) there were slowed or decreased apical endocytosis of ligand compared to the basolateral endocytosis.

If anti-SC Fab fragments did not bind to the apical surface, or bound but were not endocytosed, the unidirectionality of transcytosis could be easily explained. Basolaterally inter-

nalized ligand could be randomly delivered to either the apical or basolateral surfaces; however, the cohort delivered to the apical surface could not return to the basolateral surface if apically exposed pIg-R did not bind ligand and/or there were no endocytosis of ligand.

As discussed above, there is a substantial amount of Fab binding at the apical surface (see Table I). We then determined the amount of internalization of anti-SC Fab fragments at the apical surface. MDCK cells that express the pIg-R were preincubated for 2 h in the absence or presence of 0.1 mg/ml leupeptin. Radio-iodinated anti-SC Fab fragments were bound at 4°C for 2 h to the apical surface. Subsequently, the apical surface was washed for 60 min to remove nonspecifically bound Fab fragments. The cells were rapidly warmed to 37°C for 5 min to allow apical internalization, and then cooled to 4°C. Cells were then exposed to gentle proteolysis at the apical surface (as described above). The amount of radioactivity in the protease-sensitive (cell surface) and protease-resistant (intracellular or internalized) fractions were determined. The entire experiment was performed in the absence or presence of 0.1 mg/ml leupeptin.

Approximately 55% of apically bound Fab fragments became protease resistant (therefore internalized) during the 5-min incubation at 37°C, in the absence or presence of leupeptin. This is 70% of the amount of internalization that occurs over 5 min at the basolateral surface. Since there is a significant amount of apical binding and only a slight decrease in the amount of internalization of anti-SC Fab fragments at the apical surface, we feel that it is unlikely that these factors significantly contribute to the unidirectionality of transcytosis.

Finally, an enhanced rate of dissociation from intact receptor at the apical surface as compared to basolateral might also contribute to unidirectionality of transcytosis. We have measured the dissociation of ligand at 37°C at both surfaces (see above, Fig. 6). We found them to be comparable except at very early time points where there appears to be slightly more dissociation at the apical surface. Taken together, the slightly faster rate of ligand dissociation at the apical surface and the slightly decreased amount of endocytosis at the apical surface (at least at 5 min), may explain why only 55% of apically bound ligand is internalized over 5 min whereas 80% of basolaterally bound ligand is internalized over the same time period.

Fate of Apically Internalized Anti-SC Fab

The observed differences in the rates of dissociation at the apical and basolateral surfaces do not seem large enough to account for the unidirectionality of transcytosis. This suggests to us that factors in addition to those operating at the cell surface (i.e., internalization and dissociation) help determine the fate of ligand internalized by the pIg-R. In addition, if the fate of internalized ligand were simply the result of the relative rates of ligand dissociation and internalization at the apical versus basolateral surface, it might be predicted that the fate of ligand internalized from either the basolateral or the apical surface would be the same.

The fate of an apically internalized cohort was therefore determined. As above, anti-SC Fab fragments were bound to the apical surface of MDCK cells that express the pIg-R. After extensive washing, cells were warmed to 37°C for 5 min to allow internalization. After cooling to 4°C, the apical surface was exposed to gentle proteolysis (described above) and again warmed to 37°C. At various times, the apical and basolateral medium was sampled for the appearance of ligand. Each sample was TCA precipitated to determine the amount of intact and degraded anti-SC Fab fragments that appeared in the medium. The entire experiment was performed in the absence or presence of 0.1 mg/ml leupeptin.

In the absence of leupeptin (Fig. 7 A), 50% of internalized Fab fragments are returned intact to the apical medium over 90 min. Only 5% is delivered intact to the basolateral medium. A small fraction of internalized ligand is degraded and delivered equally to the apical and basolateral medium (~2% each). The remainder (40%) is cell associated at the end of this time course. We do not know why this material is still cell-associated. It may be in a slow recycling pool or slowly delivered to other destinations (Simmons and Schwartz, 1984). Similar results are obtained in the presence of leupeptin (Fig. 7 B). 35% of internalized Fab fragments are returned to the apical surface intact; whereas 5% is transported to the basolateral medium. Again, only a small fraction of internalized Fab fragments are degraded. Thus, apically internalized anti-SC Fab fragments have primarily one fate (return to the apical surface).

What factors could determine this fate? Both the enhanced rate of internalization at the basolateral surface and the decreased rate of dissociation of ligand at the basolateral surface (versus apical) favor the apical release of apically internalized ligand. Thus, no intracellular sorting needs to be invoked to explain the fate of apically internalized ligand. However, basolaterally internalized molecules have two fates (Fig. 2) as opposed to apically internalized molecules, which have primarily one fate (Fig. 7). This suggests that the relative rates of internalization and dissociation of ligand at the apical and the basolateral surfaces do not entirely determine the fate of internalized ligand.

Discussion

The pIg-R serves as a useful model system for the study of protein traffic and epithelial cell polarity. The pIg-R undergoes several sorting events on its itinerary through the polarized epithelial cell. We have focused in this report on the sorting events and pathway of the ligand once receptor and ligand have been internalized.

It has been assumed that after internalization at the basolateral surface of epithelial cells the pathway of ligand is a one-way transit from the basolateral surface to the apical surface, where ligand is released (Mostov and Simister, 1985). This transcytosis pathway was thought to be the only fate of internalized ligand. In this study, we have directly addressed this issue. By using anti-SC Fab fragments as ligand and using gentle proteolysis of the basolateral surface of MDCK cells, we have been able to biochemically isolate a single cohort of basolaterally internalized ligand. This allows us to demonstrate that this cohort has two fates. One was the expected, transcytosis to the apical surface and release into the apical medium. However, we also observed a significant amount (45%) of basolaterally internalized ligand that recycles to the basolateral surface. As previously outlined, ligand might be recycling with receptor (receptor mediated) or in fluid phase (after dissociation from the receptor in an en-
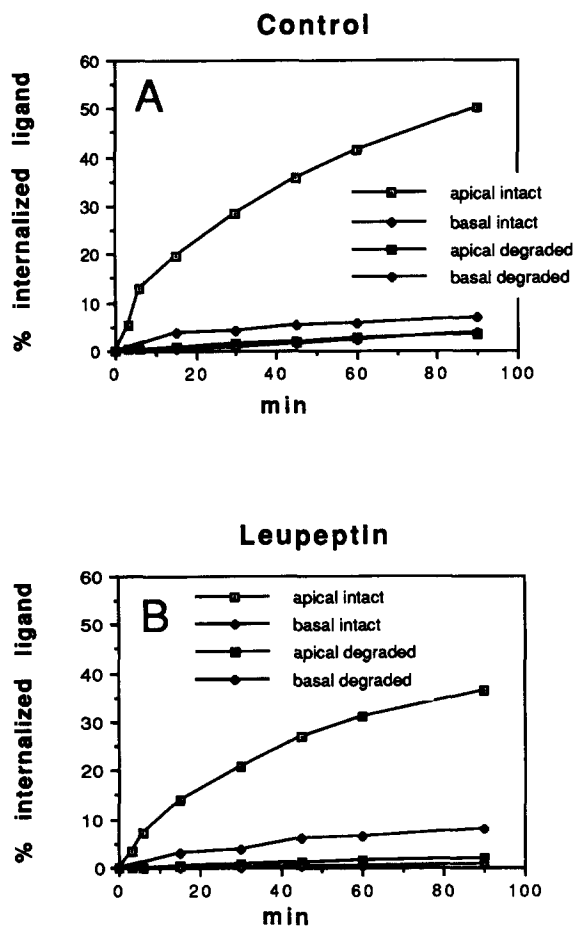
Control

Leupeptin

Figure 7. Fate of apically internalized ligand. Cells cultured on suspended filters were treated for 2 h without or with 0.1 mg/ml leupeptin at 37°C. [^{125}I]Anti-SC Fab fragments (4 μg/ml) were bound at the apical surface for 2 h and apical surface was extensively washed. The cells were rapidly warmed to 37°C for 5 min and rapidly cooled to 4°C. The apical surface was exposed to a mixture of 50 μg/ml chymotrypsin and 50 μg/ml proteinase K for 60 min at 4°C. After washing, cells were rewarmed to 37°C, and the apical and basolateral medium was collected at the times indicated. The entire experiment was performed in the absence (*A*) or presence (*B*) of 0.1 mg/ml leupeptin. Samples were TCA precipitated and the soluble and insoluble material was counted in a gamma counter. Points were performed in duplicate, which varied by 10% or less of the average value. Values are expressed as a percentage of the total amount of ligand internalized at the apical surface during the initial 5-min incubation at 37°C (6,960 cpm in the presence of leupeptin, and 3,990 cpm in the absence of leupeptin). The increase in the absolute amount of internalization in the presence of leupeptin was the result of increased binding of ligand at the apical surface.

dosomal compartment). Thus, our data do not directly address receptor recycling.

These observations regarding the postendocytotic fate of basolaterally internalized pIg-R ligand are in contrast to another system studied in MDCK cells, the transferrin receptor. Fuller and Simons (1986) have characterized the polarity of the endogenous transferrin receptor MDCK cells and found that nearly all surface transferrin receptors are located basolaterally. In addition, they noted that, after internaliza-

tion, the transferrin receptor and its ligand recycle to the basolateral surface with an accuracy of >99.8%. Therefore, with the transferrin receptor, the basolateral postendocytotic pathway of its ligand involves one pathway exclusively. The observation that internalized ligand follows more than one pathway is true for other receptor–ligand systems. For example, the majority of internalized asialoglycoproteins (>70%) are sorted to the lysosome. However, up to 28% of internalized ligand can follow at least two separate postendocytotic, recycling pathways (Simmons and Schwartz, 1984).

What drives the unidirectional transcytosis of the pIg-R and its ligand? As mentioned in the introduction, unidirectionality could be achieved by confining the protease that converts the receptor to SC to the apical surface. This mechanism is analagous to that proposed for the transport of monomeric IgG from the apical to the basolateral surface of enterocytes in the proximal small intestine of newborn rats. In this system, the IgG receptor (Simister and Rees, 1985) binds IgG at pH 6 (the pH of the neonatal intestinal lumen and thus the apical surface of intestinal epithelium) but is released at pH 7.5 (that of blood and thus the basolateral surface of the intestinal epithelium). Hence, it has been proposed that the pH gradient itself could drive the polarity of the pathway from the apical surface to the basolateral surface (Waldmann and Jones, 1973; Rodewald and Kraehenbuhl, 1984).

To test whether or not cleavage to SC drives transcytosis of pIg-R and its ligand, we inhibited the protease with leupeptin. We have demonstrated that, in MDCK cells that express the pIg-R by virtue of retroviral transfer, leupeptin can effectively inhibit the cleavage of the receptor and release of SC into the apical medium in a dose-dependent manner. In addition, leupeptin greatly prolongs the half-life of the receptor. Despite the lack of release of SC into the apical medium in the presence of leupeptin, transcytosis of ligand from the basolateral to the apical medium is not impaired. Additionally, recycling of ligand to the basolateral surface is also not affected by the presence of leupeptin. This data strongly suggests that the unidirectionality of transcytosis of ligand is not driven by the proteolytic cleavage of the pIg-R.

Unidirectional transcytosis from the basolateral to the apical medium might also be achieved by slowed or decreased endocytosis and/or enhanced dissociation of ligand from receptor at the apical surface compared to the basolateral surface. These models require no intracellular sorting of internalized ligand and assume random delivery of internalized ligand to either surface. Directionality is then determined by the relative rates of dissociation and internalization of ligand from the various surfaces. We investigated these possibilities and found that there is a substantial amount of apical endocytosis of anti–SC Fab fragments (70% of that found basolaterally) and that anti–SC Fab fragments do spontaneously dissociate from the pIg-R at both surfaces (although somewhat faster from the apical surface). Both observations favor apical release of internalized ligand randomly delivered to both surfaces, and might explain the unidirectionality of transcytosis, at least in part.

However, we found that an isolated cohort of ligand internalized from the basolateral surface has two major fates, with slightly more ligand being released basolaterally than apically (45% versus 30%). Similarly, we determined the fate

of an apically internalized cohort of ligand. This internalized cohort almost exclusively returned to the apical surface. If the fate of internalized ligand was determined only by the relative rates of dissociation and internalization at the two surfaces, we would expect the fate of internalized ligand would be the same regardless of the surface from which it was internalized. Since we have found a major difference in the postendocytotic fate of ligand endocytosed from the basolateral versus the apical surface, this argues against such a model.

We suggest that the major difference in the postendocytotic fate of ligand endocytosed from the basolateral versus the apical surface is consistent with differential intracellular sorting and that this may account for the unidirectionality of transcytosis. Ligand internalized from the basolateral surface has two major fates. It can be transported directly to the apical surface or recycle to the basolateral surface, where it is available for further rounds of endocytosis. Ligand that reaches the apical surface can either be released into the apical medium (by cleavage of pIg-R to SC or by dissociation from receptor without receptor cleavage) or it can be endocytosed. However, ligand that is endocytosed at the apical surface has only one major fate: recycling to the apical surface. It cannot return to the basolateral surface. This "trapping" of ligand in an apical recycling pathway, which does not permit return to the basolateral surface, may be a major determinant of unidirectional transcytosis. Bartles et al. (1987) have shown that apically targetted rat hepatocyte proteins arrive at the basolateral surface prior to delivery to the apical surface. This pathway may be analagous to the transcytosis of ligand for the pIg-R. Thus, the targeting of apical membrane proteins in the rat hepatocyte may involve a similar trapping mechanism. In fact, differences in postendocytotic fate may be a general mechanism that preserves the distinct compositions of the two surfaces of epithelial cells (Fuller and Simons, 1986).

Why is the postendocytotic pathway of ligand endocytosed from the two sides of the cell so different? One possibility is that this difference is nonspecific sorting (by default) that occurs without any sorting signals. It may be that the pathways we have observed reflect the bulk flow of membrane proteins (or fluid phase markers, if the ligand dissociates from receptor after endocytosis (von Bonsdorff et al., 1985)) endocytosed from the two surfaces. Alternatively, the two pathways may be intrinsically different in regard to their sorting of the pIg-R and its ligand. Basolaterally internalized receptor-ligand complexes may be sorted by different cellular machinery from apically endocytosed complexes. Finally, the pIg-R may express a signal(s) that results in this postendocytotic sorting. In support of this, we have generated mutations in the pIg-R that lead to alterations in the postendocytotic fate of both receptor and ligand (Casanova et al., manuscript in preparation). One such mutation delivers a substantial fraction of basolaterally internalized ligand and receptor to lysosomes for degradation, while another mutation causes the vast majority of basolaterally internalized ligand to follow the recycling pathway. This indicates that ligand fate is dependent on receptor structure. We speculate that the signal for unidirectional transcytosis may be a conformational change, or a posttranslational modification of the pIg-R.

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