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# Protein traffic in polarized epithelial cells: the polymeric immunoglobulin receptor as a model system

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## SUMMARY

**As a model system to study protein traffic in polarized epithelial cells, we have used the polymeric immunoglobulin receptor. This receptor travels first to the basolateral surface, where it can bind polymeric IgA or IgM. The receptor is then endocytosed and delivered to endosomes. The receptor is sorted into transcytotic vesicles, which are exocytosed at the apical surface. The 103-amino acid cytoplasmic domain of the receptor contains several sorting signals. The 17 residues closest to the membrane are an autonomous signal that is necessary and sufficient for basolateral sorting. For rapid endocytosis there are two independent signals, both of which contain critical tyrosine residues. Finally, transcytosis is signaled by phosphorylation of a particular serine.**

Key words: transcytosis, protein sorting, sorting signals

## INTRODUCTION

The most basic type of organization of cells into tissues is that of epithelia (Simons and Fuller, 1985; Rodriguez-Boulan and Nelson, 1989). Epithelial cells line a cavity or cover a surface. As such, they can form a selective barrier to the exchange of molecules between the lumen of an organ and an underlying tissue. For many decades physiologists have studied the movements of small molecules such as water, ions or sugars across epithelia. It has become increasingly clear that large molecules, such as proteins, can also cross an epithelial layer. One way that this can occur is by diffusion between cells, i.e. by a paracellular route. However, in many types of epithelia, the cells are closely attached to each other by tight junctions. These tight junctions form an effective seal between cells and usually preclude paracellular transport of macromolecules (Cereijido et al., 1989).

The other, more common transport route is across the cells themselves via vesicular carriers, in a process known as transcytosis (Mostov and Simister, 1985; Bomsel and Mostov, 1991). Macromolecules enter the cell by endocytosis, whereby a small region of the plasma membrane invaginates and pinches off, forming a vesicle. The encapsulated molecules are then transported across the cell. When these vesicles reach the opposite side, they fuse with the plasma membrane and their contents are released by exocytosis. Transcytosis may occur in either direction across a cell, from apical-to-basolateral, or basolateral-to-apical.

Transcytosis is a particularly complex example of the general process of membrane traffic in epithelial cells, and it is useful to consider transcytosis in relation to other protein transport processes that have been studied in polarized cells (Simons and Wandinger-Ness, 1990; Bomsel and Mostov, 1991). One such transport process is the targeting of membrane proteins to different poles of the cell. In most epithelial cells, the apical and basolateral surfaces maintain different protein and lipid compositions. Several mechanisms are involved in establishing and maintaining this compositional asymmetry (Bomsel and Mostov, 1991). First, newly synthesized plasma membrane proteins can be targeted directly to the appropriate membrane domain. In Madin-Darby canine kidney (MDCK) epithelial cells, segregation of apical and basolateral proteins appears to take place in the *trans*-most Golgi compartment or *trans*-Golgi network (TGN) (Griffiths and Simons, 1986). From here, proteins are packaged into vesicles destined for either the apical or basolateral surface, as demonstrated in work on viral coat membrane proteins (Rindler et al., 1984).

A second mechanism for the maintenance of cell polarity is the resorting of membrane proteins following endocytosis from the cell surface (Brown et al., 1983; Bomsel and Mostov, 1991). Internalized proteins can be transported to any of a number of cellular destinations. (1) They can be recycled to the plasma membrane from which they were internalized. Examples of this class of protein include the transferrin and LDL receptors (Brown et al., 1983). (2) They can be transported to lysosomes for degradation (for example, EGF receptor). (3) They can enter the transcytotic pathway for transport to the opposite pole of the cell. In hepatocytes, these three classes of proteins have been shown to segregate in an early endosomal compartment (Geuze et al., 1984). This review focuses on the cellular and molecular mechanisms of IgA transport across epithe-

lial cells, but we can only cover selected aspects of this problem and will concentrate primarily on research done by our laboratory over the last decade.

## TRANSCYTOSIS

A wide variety of molecules have been shown to be transcytosed (Mostov and Simister, 1985; Bomsel and Mostov, 1991), and it is likely that transcytosis is ubiquitous in epithelia. The best-studied examples are transport of immunoglobulins that occurs in at least three situations in mammals: transport of IgA and IgM across various mucosa (Childers et al., 1989), transport of IgG across the intestinal epithelium in newborn rats (Rodewald and Kraehenbuhl, 1984) and transport of IgG across the human placenta. The first process is discussed in detail below. Other examples of transcytosis include: insulin and serum albumin across endothelia (King and Johnson, 1985), epidermal growth factor across kidney epithelia (Maratos-Flier et al., 1987), nerve growth factor across intestinal epithelium and transferrin across capillaries in the brain (Bomsel and Mostov, 1991).

In some cases, transcytosis is a quantitatively major pathway. In hepatocytes, for example, newly synthesized apical (bile canalicular) membrane proteins are not delivered directly to the apical surface (Bartles et al., 1987); rather these proteins are first sent to the basolateral (sinusoidal) surface and from there transcytosed to the apical plasma membrane. In these cells, transcytosis is the only way for membrane proteins to reach the apical surface. In the intestinal cell line, CaCo2, a number of apical proteins utilize both the direct TGN-to-apical pathway and the indirect transcytotic pathway (LeBivic et al., 1990; Matter et al., 1990) to reach the apical surface.

## TRANSCYTOSIS OF POLYMERIC IMMUNOGLOBULINS

The major class of immunoglobulin found in a wide variety of mucosal secretions, such as gastrointestinal and respiratory secretions, is IgA (Brandtzaeg, 1981; Bienenstock, 1984; Ahnen et al., 1985; Childers et al., 1989). IgA is produced by submucosal plasma cells that are often found in lymphoid aggregates such as gut-associated lymphoid tissue (GALT) and bronchus-associated lymphoid tissue (BALT) (Bienenstock, 1984). After secretion, IgA is taken up by an overlying epithelial cell, transported across the cell and released into external secretions (Brandtzaeg, 1981). Here the IgA forms the first specific immunologic defence against infection. This transport system transports only polymeric immunoglobulins (Brandtzaeg, 1981). Dimers or higher oligomers of IgA are transported, as are pentamers of IgM, although transport of the latter is less efficient. All of these polymers contain the J (joining) chain.

This transport of polymeric immunoglobulins is now known to be a receptor-mediated event. In 1965, Tomasi and associates found that IgA isolated from human saliva contained an extra polypeptide that was a glycoprotein of 70 kDa (Tomasi et al., 1965). This protein, named secretory component (SC), was found to be synthesized by epithelial cells and added to IgA as it was transported across the cell. In 1974, Brandtzaeg examined the cellular location of SC by immunofluorescence (Brandtzaeg, 1974) and reported its presence on the basolateral surface of various epithelial cells. IgA from appropriate secretions could bind to the basolateral cell surface, and antisera to SC could block the binding of IgA. This led to the proposal that SC acted as a receptor mediating the uptake and transport of IgA across cells.

The hypothesis that SC was an IgA receptor presented an interesting paradox that formed the basis for many studies (Brandtzaeg, 1974; Mostov et al., 1980). If SC were a receptor for IgA on the basolateral cell membrane, it would be expected to be an integral membrane protein that could only be solubilized with detergents, yet SC isolated from secretions was water-soluble and had no affinity for membranes. One proposed solution to this paradox was that SC was secreted at the basolateral surface and combined with IgA in the extracellular fluid or blood (Kuhn and Kraehenbuhl, 1979). The SC-IgA complex could then bind to an unidentified receptor on the basolateral cell surface and be transported to the luminal surface. A problem with this model was that no SC could be detected in blood.

An alternative hypothesis was that SC was part of a larger precursor, now known as the polymeric immunoglobulin receptor (pIg-R) (Mostov et al., 1980). The first evidence in support of this model came from cell-free translation of rabbit liver and mammary mRNA (Mostov et al., 1980). SC was found to be synthesized as a large precursor of about 90 kDa. In a cell-free membrane integration system, this precursor was shown to be a membrane-spanning protein, which had a cytoplasmic domain of about 10 to 15 kDa. Moreover, the precursor could specifically bind to IgA, suggesting that it was the true polymeric immunoglobulin receptor (pIg-R).

We next studied the biosynthesis and processing of pIg-R in a human colon carcinoma cell line, HT29, which was known to secrete SC (Mostov and Blobel, 1982). Biosynthetic labelling of these cells revealed that the pIg-R was found initially as a single species of 90 kDa. Its carbohydrate side chains were subsequently modified to the complex type, which increased the apparent size of the protein to about 105 kDa. The pIg-R was then slowly cleaved to SC (70 kDa) and released from the cells. This cleavage is slower than in vivo, probably because the HT29 cells are not well differentiated or polarized. This type of pulse-chase analysis has been carried out by others using rabbit mammary cells in culture and intact rat liver, and the general observations have been confirmed (Solari and Kraehenbuhl, 1984; Sztul et al., 1985a,b).

The current understanding of the general pathways taken by the pIg-receptor is summarized in Fig. 1. An epithelial cell is shown with the apical surface at the top and the basolateral surface at the bottom. In step 1, the pIg-R is synthesized by membrane-bound polysomes of the rough endoplasmic reticulum (RER) as an integral membrane protein. A portion of the molecule extends into the lumen of the RER (open circle), a segment spans the membrane, and a portion is in the cytoplasm (filled circle). After transport through the Golgi apparatus (step 2), the receptor is tar-

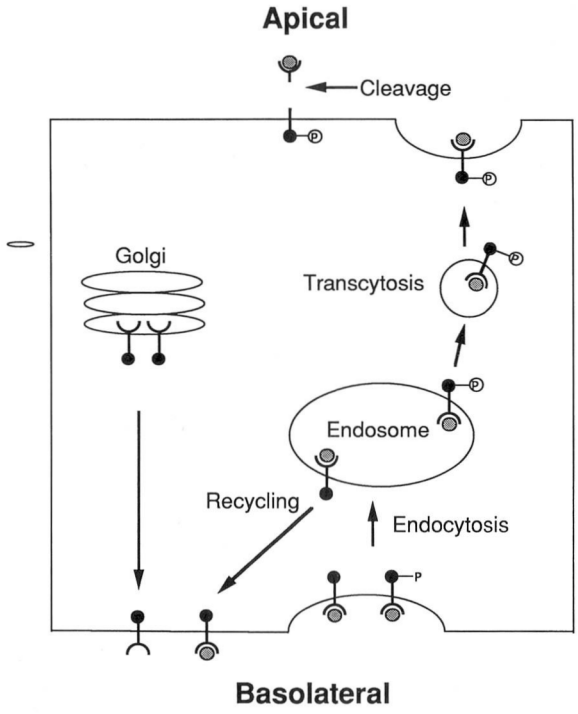


**Fig. 1.** The pathway of IgA transcytosis. An epithelial cell is shown, with the apical surface at the top and the basolateral surface at the bottom. Junctional complexes divide the two surfaces and join the cell to its neighbors. For further details see text.

geted to the basolateral surface (step 3). Here the portion of the molecule formerly in the RER lumen is outside the cell, where it can bind IgA (step 4). The receptor-ligand complex is then endocytosed in coated vesicles (step 5) and transported by a variety of vesicles and tubules to the apical surface (step 6). At the apical surface (or perhaps during transport), the extracellular portion of the pIg-R is proteolytically cleaved from the transmembrane anchor. This cleaved fragment is the previously identified SC. It remains associated with the IgA in the extracellular secretions, and has the additional function of stabilizing the IgA against denaturation or proteolysis in the harsh external environment.

Our next step was to clone cDNA for pIg-R (Mostov et al., 1984). We used rabbit liver mRNA, an abundant source of pIg-R mRNA. We obtained a full-length cDNA clone that encoded a protein of 755 amino acids (not including the 18-amino acid N-terminal signal sequence). The protein had a single membrane-spanning segment and a cytoplasmic C-terminal domain of 103 amino acids (Fig. 2). The extracellular ligand-binding portion, which is cleaved to generate SC, contains five homologous repeating domains of 10 to 110 residues each. These are members of the immunoglobulin superfamily, and most closely resemble immunoglobulin variable regions (Mostov et al., 1984).

In the rabbit, there appears to be only one gene for pIg-R (Deitcher and Mostov, 1986). However, in many rabbits, there are four primary translation products, two of 70 to 75 kDa and two of 90 to 95 kDa. Part of the heterogeneity is due to the existence of multiple alleles of rabbit SC and

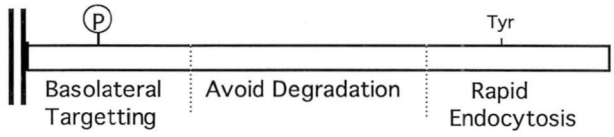


**Fig. 2.** Arrangement of sorting signals in the cytoplasmic domain of the pIg-R. The membrane is at the left and the carboxy terminus is at the right. The phosphate group is indicated by the P. For further details see text.

most rabbits are heterozygous. However, even in homozygous rabbits, there are two primary translation products, one of 70 to 75 kDa and one of 90 to 95 kDa. The pIg-R mRNA can be alternately spliced to yield a pIg-R protein that lacks the second and third of the five immunoglobulin domains (Deitcher and Mostov, 1986). This alternately spliced form is the 70 to 75 kDa translation product, whereas the form with all five immunoglobulin domains is the 90 to 95 kDa product. Sequencing of a genomic clone revealed that the two immunoglobulin domains involved are encoded by a single large exon. This is a rare example of two immunoglobulin domains encoded on one exon.

## EXPRESSION OF pIg-R cDNA

The pathway of the pIg-R is unusual for a membrane protein in polarized cells because it is targeted first to the basolateral surface and then, following endocytosis, to the apical surface, rather than directly to its final destination. Postendocytotic sorting is complicated by the fact that internalized pIg-R enters endosomes that contain a variety of other receptors and ligands (Geuze et al., 1984). The pIg-R is apparently sorted away from this mixture of receptors and ligands in tubular extensions of the endosomes, and is then packaged to carrier vesicles for transcytosis to the apical surface (Geuze et al., 1984). These two features of the pIg-R pathway, sequential targeting to both surfaces and postendocytotic sorting into the transcytotic pathway, offer unique opportunities for the study of membrane trafficking. Our major strategy in studying the sorting of the pIg-R has been to express the cloned rabbit pIg-R cDNA in cultured cell lines, and analyze the function of normal and mutant receptors. For expression we have used a retroviral vector system developed by Mulligan and colleagues (Breitfeld et al., 1989a).

First, the pIg-R cDNA was expressed in the fibroblast line, y2, which is a derivative of the NIH 3T3 mouse fibroblast line (Deitcher et al., 1986). Although these cells have no obvious apical-basolateral polarity, the pIg-R appears to function normally. In a pulse-chase experiment, the receptor was found to be first synthesized as a single species of about 90 kDa. In the subsequent chase, it was converted to a heterogeneous form of about 105 kDa, due to modification of the carbohydrates, and was eventually cleaved to release SC, which was released into the medium. Like authentic SC, the SC released by these cells was heterogeneous. This is apparently due both to heterogeneity in glycosylation and to heterogeneity in the exact site of cleavage from the transmembrane anchor.

In these fibroblasts, when the pIg-R reaches the cell surface it is not immediately cleaved to SC. This results in a pool of uncleaved receptor on the surface where it is capable of internalizing bound ligand. About 35% of the bound ligand can be internalized. This endocytosed ligand is rapidly recycled back to the surface and then released into the medium, without substantial degradation.

We next expressed the pIg-R in cultured epithelial cells (Mostov and Deitcher, 1986), specifically, the Madin-Darby canine kidney (MDCK) cell line, which has been widely used for studying cell polarity. When grown on porous filter supports, such as a Millipore filter, these cells form a well-polarized, electrically resistant epithelial monolayer (Simons and Fuller, 1985). Tight junctions separate the apical from the basolateral surfaces and, in effect, a simple epithelial tissue is reconstituted in culture. The monolayer is relatively impermeable, especially to macromolecules. Under these culture conditions, one can experimentally access either the apical surface or, through the filter, the basolateral surface. In MDCK cells, as in fibroblasts, pIg-R is synthesized as a single species and then converted to a heterogeneous form due to carbohydrate modification. Proteolytic cleavage also occurs in these cells, and the free SC is released almost exclusively into the apical medium. This is exactly as occurs in vivo: SC is released at the lumenal surface and not into the bloodstream. Moreover, if $^{125}$I-labeled dIgA is added to the basolateral medium, it is taken up by the cells and transported into the apical medium. This transcytosis is saturated by a competing excess of unlabeled dIgA, and does not occur in cells that do not express the receptor. Most importantly, the transport of dIgA is unidirectional, occurring only in the basolateral-to-apical direction. This process occurs with a half-time of about 30 minutes (Mostov and Deitcher, 1986).

## REGULATION OF SORTING OF pIg-R

We have carried out additional studies to determine why the pathway for transcytosis of pIg-R is unidirectional (Breitfeld et al., 1989c). One possibility is that unidirectionality is conferred by the protease that cleaves the pIg-R to SC at the apical surface. To test this hypothesis, we took advantage of the recent observation that the cleavage of pIg-R to SC is inhibited by the microbial thiol protease inhibitor, leupeptin (Musil and Baenziger, 1987). We found that even though cleavage to SC was inhibited, transcytosis of ligand to the apical surface and its release into the apical medium were unaffected. In the absence of cleavage, ligand simply dissociates from the uncleaved pIg-R at the apical surface. More importantly, if ligand was added to the apical medium, apical-to-basolateral transcytosis was not observed. These results indicate that the unidirectionality of transport is conferred by signals other than proteolytic release of SC.

It appears that transcytosis is imperfect in terms of its efficiency. When a ligand molecule is endocytosed from the basolateral surface, it has three possible fates: transcytosis to the apical surface, recycling to the basolateral surface, or degradation. We have recently developed an assay that allows us to examine the fate of ligand endocytosed at the basolateral surface (Breitfeld et al., 1989c). We found that about 55% of internalized ligand is transcytosed over a 2 hour time course, while about 20% recycles. Very little (3-5%) is degraded. The recycling of receptor to the basolateral surface provides a further opportunity to be re-endocytosed and transcytosed. We also found that ligand could be endocytosed from the apical plasma membrane (Breitfeld et al., 1989c), but that this apically internalized ligand mostly recycles back to the apical surface. Almost none is transcytosed to the basolateral domain. There is thus a clear difference between ligand endocytosed from the basolateral surface, which can go to either surface, and ligand endocytosed apically, which can only return to the apical domain. It appears that once the receptor reaches the apical plasma membrane, it is essentially 'trapped'; it is then either cleaved to SC, or endocytosed and recycled back to the apical surface. The molecular signals that account for these observations are not known.

It is interesting to note that treatment of the MDCK cells with the microtubule-depolymerizing drug nocodazole slows the rate of transcytosis by 60-70%, but does not affect the overall accuracy of delivery (Breitfeld et al., 1990a; Hunziker et al., 1990). This suggests that microtubules may facilitate the process of transcytosis, but are not absolutely required for targeting of vesicles to the appropriate membrane. Importantly, transport of newly synthesized receptor from the Golgi to the basolateral membrane is completely unaffected by nocodazole treatment, suggesting that this process does not involve microtubules.

## SORTING SIGNALS IN THE pIg-R

A major goal of our research is to analyze the structural determinants inherent to molecules, such as the pIg-R, that direct them to the appropriate cellular locations. We assume that the pIg-R contains sorting signals that control its transport. The complexity of the pIgR's cellular itinerary suggests that it may contain multiple sorting signals. One obvious location for such signals is the 103-amino acid, C-terminal cytoplasmic domain. Being in the cytoplasm, this receptor 'tail' would be accessible to interact with proteins in the cytoplasm that presumably constitute the cellular sorting machinery.

To address this issue, we first constructed a mutant pIg-R that lacked 101 of the 103 amino acids of the cytoplasmic domain (Mostov et al., 1986) (Fig. 2). Oligonucleotide-directed in vitro mutagenesis was used to insert a stop codon two amino acids after the membrane-spanning segment. This truncated 'tail-minus' receptor was expressed using the retroviral vector system in both nonpolarized fibroblasts and in MDCK cells.

In fibroblasts, the receptor is transported normally to the surface and is cleaved to SC. However, unlike the wild-type pIgR, the tail-minus mutant is not endocytosed, an observation that is not completely surprising. In other systems, notably the low density lipoprotein receptor, the cytoplasmic tail has been shown to be essential for endocytosis (Davis et al., 1987). When expressed in MDCK cells, this tail-minus pIg-R does not appear at the basolateral surface, rather it is sent directly to the apical surface from the

Golgi and is cleaved to SC. This result suggests that the cytoplasmic domain either contains a signal for basolateral targeting or that the cytoplasmic domain is necessary for basolateral targeting to occur. In a separate construction, we further truncated the receptor by deleting both the transmembrane and cytoplasmic domains, producing a soluble receptor (Mostov et al., 1987) (Fig. 2). This 'anchor-minus' receptor was secreted predominantly from the apical pole of MDCK cells. This suggests that the extracellular (or luminal) portion of the pIg-R may contain an apical sorting signal.

Recently we have made a large number of mutations in the cytoplasmic domain of the pIg-R, which indicate that it contains at least four sorting signals (Breitfeld et al., 1989b; Bomsel and Mostov, 1991). The arrangement of these signals is depicted in Fig. 2. Deletion of the carboxy-terminal 30 amino acids, which are encoded by a single exon, produces a receptor that follows the pathway of the wild-type receptor, except that the rate of endocytosis from the basolateral surface is decreased by about 60% (Breitfeld et al., 1990b). Exactly the same phenotype is produced by mutation of a tyrosine residue in this segment to a serine. This result is consistent with previous observations in several other systems, which have shown that tyrosine residues are important for rapid, clathrin-mediated endocytosis (Davis et al., 1987; Jing et al., 1990) and suggests a similar role for tyrosine in the pIgR. Mutation of a second, more membrane-proximal tyrosine reduces the endocytotic rate by only 5-10%. However, mutation of both tyrosines together virtually eliminates endocytosis, suggesting that both residues may play a role in this process.

Deletion of 37 residues from the middle section of the tail produces a pIg-R that is endocytosed normally, but is then degraded (Breitfeld et al., 1990a). It is unlikely that this receptor is simply malfolded, as it is normally delivered to the basolateral cell surface, binds ligand and is endocytosed. Perhaps the receptor has a mechanism to avoid degradation, which has been disrupted by the mutation. Alternatively, a signal for degradation may have been artificially created.

Further mutational analysis indicates that only the 17 amino acids closest to the membrane are needed for basolateral targeting (Casanova et al., 1991). A truncated receptor containing only these residues in the cytoplasmic domain is sorted basolaterally from the TGN, while deletion of these residues, leaving the remainder of the tail intact, produces a receptor that is targeted directly to the apical surface. Moreover, transplantation of this 17-amino acid signal to a heterologous, normally apical protein (placental alkaline phosphatase) is sufficient to redirect the chimeric protein to the basolateral surface.

Finally, the receptor has been shown to be phosphorylated on a serine residue in the cytoplasmic domain (Larkin et al., 1986). Phosphorylation occurs at the basolateral surface and/or shortly after endocytosis. Mutation of this serine to an alanine, which cannot be phosphorylated, produces a receptor that is not efficiently transcytosed, but rather recycles to the basolateral surface (Casanova et al., 1990). In contrast, mutation of this serine to an aspartic acid, whose negative charge may mimic that of the phosphate group, produces a receptor that is transcytosed more efficiently than wild type. These results indicate that phosphorylation is the signal that directs the segregation of receptor into the transcytotic pathway.

The aspartic acid mutant also allows several indirect conclusions to be drawn. First, if the function of the pIg-R were simply to maximally transcytose IgA, why would the cell use phosphorylation, rather than simply using aspartate at this site? The most likely explanation is that phosphorylation is used to regulate transcytosis, perhaps in response to external cues. Second, many other proteins are transcytosed in epithelial cells. These may not necessarily use a phosphorylation mechanism, but may instead be analogous to the Asp mutant. Third, both the TGN and the basolateral endosome are organelles where apical/basolateral sorting takes place. However, the endosome relies on a negative charge from either phosphate or aspartate at a specific site in the pIg-R to send the molecule to the apical surface. The TGN, in contrast, ignores this charge, and sends even the Asp mutant to the basolateral surface. In other words, the negative charge does not inactivate the signal for TGN to basolateral targeting. Rather, it appears that targeting from the TGN and the endosome use different mechanisms.

Having made substantial progress in defining these sorting signals, the next step is to analyze the cellular machinery that recognizes such signals and is ultimately responsible for sorting processes. A first step in this direction is the work of Sztul and colleagues (Sztul et al., 1991), who have isolated putative transcytotic vesicles from rat liver. These vesicles are enriched in a 108 kDa protein which is apparently bound to the cytoplasmic face of the vesicular membrane. The possibility that this protein may be involved in either docking of the vesicle to its target membrane or in attachment to microtubules for transport is currently under investigation.

## CONCLUSION

As this review indicates, substantial progress has been made in understanding the cellular and molecular basis of the transcytosis of immunoglobulins. This knowledge is important for two reasons. First, transcytosis is one of several systems of protein traffic in epithelial cells. Analyzing the sorting signals and cellular machinery that decode these signals will permit elucidation of the general principles that govern protein sorting. Second, transcytosis is important in the overall physiology of an organism. For example, polymeric immunoglobulins form a very early, specific immunologic defence against infection, and their transport is mediated by specific transcytotic events. Although much less is known about the regulation of their transcytosis, many other proteins are carried across epithelia by this mechanism to tissue sites where they are likely to carry out important functions. Analyzing transcytosis is thus an important area of connection between cell and molecular biology and the overall functioning of organs and organ systems.

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