Transcytosis: Crossing Cellular Barriers

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Tuma, Pamela L., and Ann L. Hubbard. Transcytosis: Crossing Cellular Barriers. *Physiol Rev* 83: 871–932, 2003; 10.1152/physrev.00001.2003.—Transcytosis, the vesicular transport of macromolecules from one side of a cell to the other, is a strategy used by multicellular organisms to selectively move material between two environments without altering the unique compositions of those environments. In this review, we summarize our knowledge of the different cell types using transcytosis in vivo, the variety of cargo moved, and the diverse pathways for delivering that cargo. We evaluate in vitro models that are currently being used to study transcytosis. Caveolae-mediated transcytosis by endothelial cells that line the microvasculature and carry circulating plasma proteins to the interstitium is explained in more detail, as is clathrin-mediated transcytosis of IgA by epithelial cells of the digestive tract. The molecular basis of vesicle traffic is discussed, with emphasis on the gaps and uncertainties in our understanding of the molecules and mechanisms that regulate transcytosis. In our view there is still much to be learned about this fundamental process.

I. INTRODUCTION

At its simplest, transcytosis is the transport of macromolecular cargo from one side of a cell to the other within a membrane-bounded carrier(s). It is a strategy used by multicellular organisms to selectively move material between two different environments while maintaining the distinct compositions of those environments. Cells have other strategies not involving membrane vesicles to selectively move smaller cargo (ions and small solutes) across cellular barriers. Paracellular transport, the movement between adjacent cells, is accomplished by regulation of tight junction permeability, and transcellular transport, the movement of ions and small molecules through a cell, is accomplished by the differential distribution of membrane transporters/carriers on opposite sides of a cell. Together, these three processes contribute to the success of multicellular organisms.
Historically, the existence of transcytosis was first postulated in the 1950s by Palade in his studies of capillary permeability (426). He described a prominent population of small vesicles, many of which were in continuity with the plasma membrane, and hypothesized that these vesicles were the morphological equivalent of the large pore predicted by the physiologist Pappenheimer to explain the high permeability of blood microvessels to macromolecules (428). N. Simionescu was the first to coin the term transcytosis to describe the vectorial transfer of macromolecular cargo within the plasmalemmal vesicles from the circulation across capillary endothelial cells to the interstitium of tissues (538). During this same period, another type of transcytosis was being discovered. Immunologists comparing the different types of immunoglobulins found in various secretions (e.g., serum, milk, saliva, and the intestinal lumen) speculated that the form of IgA found in external secretions (called secretory IgA, due to the presence of an additional protein component) was selectively transported across the epithelial cell barrier (577, 578). The pathway and origin of the component acquired during transport were actively investigated, and in 1980 secretory component (SC) in secretory IgA was identified as the ectoplasmic domain of the intestinal epithelial cell membrane receptor that binds dimeric IgA and transports it through multiple intracellular compartments to the opposite side of the cell (391, 423). These two historic transcytotic systems are still actively investigated today.

We now know that transcytosis is a widespread transport process; a variety of cell types use it, different carriers and mechanisms have evolved to carry it out, and the cargo moved by it is diverse. Cell types: we are most familiar with transcytosis as it is expressed in epithelial tissues, which form cellular barriers between two environments. In this polarized cell type, net movement of material can be in either direction, apical to basolateral or the reverse, depending on the cargo and particular cellular context of the process. However, transcytosis is not restricted to only epithelial cells. Reports of cultured osteoclasts (398, 490) and neurons (221) carrying vesicular cargo between two environments indicate that the strategy of vesicular transcytosis has been used elsewhere. Mechanisms: in intestinal cells transcytosis is a branch of the endocytic pathway, with cargo being internalized via receptor-mediated (i.e., clathrin-coated) mechanisms and progressively sorted away from internalized material destined for other cellular destinations. However, transendothelial transport in blood capillaries does not conform to this scenario, since different carriers and a more direct route are used to cross the cell. Such differences illustrate that multiple transcytotic mechanisms have evolved that depend on the particular cellular context. Furthermore, they illustrate that cargo in the transcytotic pathways seems able to avoid degradation in lysosomes. How? Cargo: the nature of the transcytotic cargo also varies. Although today we might think of transcytosis as a selective process, the originally defined system, endothelial cells of the microvasculature, moves macromolecular cargo rather nonselectively within the fluid phase of the transport vesicle or by adsorption to the vesicle membrane. Furthermore, transcytotic cargo is not limited to macromolecules. Several vitamins and ions utilize endocytic mechanisms and vesicular carriers as part of their transcellular sojourn. This brings up another unsolved mystery, that of a cell transcytosing particular cargo for use by other cells but also using some of it for its own metabolism. How is such apportionment made?

A major goal of this review is to summarize the widespread occurrence of transcytosis and focus on its many variations. First, we present documented examples of in vivo transcytosis in mammals, using the expanded definition given above. Next, we assess the status of in vitro cell models currently used to study the different types of transcytosis. We then review in more depth the two best-studied transcytosis systems, transendothelial transport of circulating macromolecules and transcytosis of IgA in polarized epithelial cells, focusing on the similarities and differences of their pathways and carriers. Finally, we present current information about the molecular mechanisms and regulation of transcytosis. Throughout, we identify gaps in our present understanding of this process, with the hope that interested researchers will fill in those gaps with insightful experiments and definitive answers.

II. DOCUMENTED TRANSCYTOSIS IN VIVO

Table 1 documents that transcytosis is widespread. As expected, epithelial cells forming barriers between the outside world and the interstitium or between the internal world (circulation) and the interstitium are the major cells participating in transcytosis. However, the question of whether transcytosis occurs in all adult epithelia (e.g., kidney and skin) is open. While proximal tubule cells are endocytically active, only micronutrients seem to be “transcytosed” by them in vivo. Other segments of the nephron, e.g., the collecting tubule, are more difficult to assess. Transcytosis certainly occurs in the most obvious fetal organs, the yolk sac and placenta, and it probably operates elsewhere in the developing fetus. Further examination of Table 1 reveals that the transport of iron, vitamin B_{12}, and the immunoglobulins IgA and IgG occurs in several organs. However, the routes and fates of the molecules are not always the same. Curiously, the routes and mechanisms by which circulating hormones gain access to their target tissues have not been extensively explored (181). Finally, although not yet examined in all polarized cells, the biogenesis of apical plasma membrane
proteins is an example of endogenous molecules using transcytosis to attain their destination.

A. Transcytosis in the Vasculature

The most extensive exchange in vivo is that of plasma constituents across the endothelium that lines the inner surface of the blood vasculature. Of the three types of endothelium, continuous, fenestrated, and discontinuous (sinusoidal), only the first two form selective cellular barriers to the passage of macromolecules between the circulation and the underlying interstitium. All continuous and fenestrated endothelia throughout the vascular system are capable of the rapid and extensive bidirectional exchange of small and large molecules, but those of the capillaries and postcapillary venules are the major players in this activity (535, 536, 539). These two parts of the vascular tree constitute what is called the microvascular exchange system, whose surface area is enormous (~600 m²) (Fig. 1A). While fenestrated endothelia are more permeable to small solutes and water than are continuous endothelia, their relative permeability to macromolecules, and hence participation in transcytosis, is controversial (534). Although obvious, it is nonetheless important to state that transcytosis is but one of many important functions carried out by vascular endothelial cells, which are dynamic and capable of rapid responses to local changes in the environment.

1. Structural features of continuous endothelium

The simple, squamous epithelial cells of continuous endothelium are quite distinctive morphologically (Fig. 1B). They are remarkably thin (0.2–0.5 μm) in regions not including nuclei. A defining feature of these and all epithelial cells is a basement membrane that underlies their basal surface (Fig. 1C). It is made collaboratively by the endothelial and underlying interstitial cells. The most prominent intracellular feature is a population of smooth-surfaced vesicles of 50–70 nm diameter, some of which are in continuity with the plasma membrane facing the circulation (the apical or luminal surface), others in continuity with the opposite surface (the basolateral or abluminal surface), and still others apparently free in the cytoplasm (Fig. 1C). These vesicles, which have an ~35-nm-diameter opening with a thin diaphragm across it, were originally termed plasmalemma vesicles but are now called caveolae (“small caves”) because of their characteristic flask shape (Fig. 1D) (10). In continuous endothelial cells, the frequency of caveolae varies widely depending on the organ. For example, in endothelium of skeletal muscle (the diaphragm), the estimate is ~1,200/μm³, whereas in pulmonary capillaries it is only ~130/μm³ (539). This variation does not correlate with permeability, suggesting other functions for caveolae. Caveolae are also found in other cell types where their functions and compositions are actively being investigated (reviewed in Refs. 9, 546).

An important feature of endothelial cells is their tight junctions, which represent a barrier to paracellular diffusion (219). While there is good experimental evidence that the permeability of endothelial cell tight junctions changes depending on local conditions (336), the molecular basis has yet to be elucidated. The discovery of a large family of tight junction membrane proteins, the claudins, and their capacity to form heterologomeric complexes with distinct permeability properties (580), will undoubtedly provide insights into the dynamic regulation of tight junction permeability in capillaries.

Important to an understanding of transcytosis in endothelial cells is the endocytic system, including clathrin-coated vesicles, endosomes, and lysosomes. These organelles are present in all capillary endothelium but are not abundant, and they are usually located in the thicker, perinuclear regions of cells. The endocytic system is clearly functional, as attested by the delivery of modified albumins and oxidized low-density lipoproteins (LDLs) to lysosomes (296, 506). But how do these cells distinguish between cargo destined for transcytosis versus that for degradation? The simplest explanation is that different cargoes use different receptors that are localized to different entry sites in the plasma membrane (PM). However, how do endothelial cells themselves utilize the same cargo that they transport for use by other cells; that is, how is the apportionment of cargo for self versus others regulated? As we shall see in section III, at least one cargo molecule (e.g., native LDL) may use different entry ports (i.e., caveolae versus clathrin-coated vesicles), offering the interesting possibility that the point of entry determines the subsequent fate of a particular internalized cargo molecule.

2. Microvascular permeability and transcytosis

Microvessels are approximately two orders of magnitude more permeable than other epithelia, making them leaky to the passage of circulating proteins into the interstitium. Most macromolecules move across capillary endothelium by bulk-phase not receptor-mediated mechanisms. Nonetheless, there is selectivity to the process, with the size and charge of cargo being important factors. Furthermore, although transport is bidirectional, the concentration gradient extending from the blood (apical side) to the interstitium (basal side) dictates that the bulk of transport is in an apical-to-basolateral direction. Finally, different continuous capillary beds have distinctive perme-selectivities, as evidenced by the varied compositions of the lymph draining from them.

The basis for high capillary permeability has been a
<table>
<thead>
<tr>
<th>Organ System</th>
<th>Cell Type</th>
<th>Cargo</th>
<th>Direction</th>
<th>Receptor/Carrier</th>
<th>Comments</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart, lung, skeletal muscle, adipose tissue</td>
<td>Continuous capillary endothelium</td>
<td>Molecules &gt; 1.7 nm Albumin</td>
<td>A-BL and BL A</td>
<td>Fluid phase in caveolae g60 in caveolae</td>
<td>Albumin plays multiple roles in caveolae-mediated transcytosis throughout the vascular tree</td>
<td>183, 378, 503, 602</td>
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<td></td>
<td></td>
<td>Orosomucoid</td>
<td>A-BL and BL A</td>
<td>Unidentified in caveolae</td>
<td></td>
<td>456</td>
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<td></td>
<td></td>
<td>IgG</td>
<td>A-BL and BL A</td>
<td>FcRn in unidentified carrier</td>
<td>Contributes to IgG homeostasis and prolonged half-life of IgG in body</td>
<td>47</td>
</tr>
<tr>
<td>Testis</td>
<td>Arterial endothelium</td>
<td>LDL cholesterol</td>
<td>A-BL</td>
<td>Fluid phase in caveolae</td>
<td>Not via LDL receptor</td>
<td>600</td>
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<td></td>
<td>Continuous capillary endothelium (has markers of brain EC)</td>
<td>Transferrin (Tf)-iron</td>
<td>A-BL</td>
<td>Non-specific in caveolae</td>
<td>Not via Tf receptor</td>
<td>236</td>
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<tr>
<td>Brain</td>
<td>Cerebral endothelium (tight continuous)</td>
<td>Insulin</td>
<td>A-BL</td>
<td>Insulin receptor via unidentified carrier</td>
<td>Rabbit, thersus monkey, binding to isolated human brain capillaries</td>
<td>133, 431</td>
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<tr>
<td></td>
<td></td>
<td>Iron</td>
<td>A-BL</td>
<td>Tf receptor via CCV</td>
<td>In vitro study, but LDL receptor present in vivo</td>
<td>112</td>
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<tr>
<td></td>
<td></td>
<td>IgG</td>
<td>BL A</td>
<td>Tf receptor via unidentified carrier</td>
<td></td>
<td></td>
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<td></td>
<td>Choroid plexus</td>
<td>Membrane associated very little A-BL; no BL</td>
<td>A-BL</td>
<td>?</td>
<td>Efflux from brain</td>
<td>501, 634</td>
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<td></td>
<td></td>
<td>Antigens, pathogens</td>
<td>A-BL</td>
<td>Both phagocytic and clathrin-mediated uptake</td>
<td>Degradation in lysosomes is predominant fate</td>
<td>590</td>
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<tr>
<td>Adult intestine</td>
<td>M cells in Peyer’s patches of ileum</td>
<td>Absorptive enterocytes</td>
<td>dlgA</td>
<td>BL A</td>
<td>pIgA receptor via CCV</td>
<td>Multiple intermediate compartments in pathway</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Newly synthesized apical PM proteins</td>
<td>Unknown</td>
<td>No intracellular intermediates identified</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Absorptive enterocytes in terminal ileum</td>
<td>Vitamin B12</td>
<td>BL A</td>
<td>Cubilin/megalin via CCV</td>
<td>See Table 2 for molecular players</td>
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<td>Liver</td>
<td>Hepatocytes</td>
<td>dlgA</td>
<td>BL A</td>
<td>pIgA receptor via CCV</td>
<td>Multiple intermediate compartments in pathway</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hepatocytes</td>
<td>Newly synthesized apical PM proteins</td>
<td>BL A</td>
<td>Unknown</td>
<td>Entry mechanisms unknown, intermediate compartments may be same as those of pIgA</td>
</tr>
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<td>Sinusoidal endothelium</td>
<td>Ceruloplasmin</td>
<td>A-BL</td>
<td>Unknown</td>
<td>Postulated to be desialylated in transit to hepatocytes</td>
<td>570</td>
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<td></td>
<td>Kidney</td>
<td>Proximal tubule cells</td>
<td>Vitamin B12</td>
<td>A-BL</td>
<td>TCII; megalin-mediated in CCV</td>
<td>Degradation of TCII in lysosomes; unknown storage site for B12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vitamin D</td>
<td>A-BL</td>
<td>D binding protein; megalin-mediated in CCV</td>
<td>Degradation of protein carrier in lysosomes; fate of vitamin D?</td>
<td>413</td>
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<td></td>
<td></td>
<td>Vitamin A</td>
<td>A-BL</td>
<td>Retinol binding protein; megalin-mediated in CCV</td>
<td>Same fate as above</td>
<td>347</td>
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<td>Neonatal intestine</td>
<td>Enterocytes</td>
<td>Maternal IgG</td>
<td>A-BL</td>
<td>FcRn via CCV</td>
<td>Subsequent movement into apical endosomes then fusion with lateral membrane</td>
</tr>
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<td></td>
<td>Yolk sac</td>
<td>Maternal B12</td>
<td>A-BL</td>
<td>Cubilin?/megalin/TCII</td>
<td>Identification of cubilin as target of teratogenic antibodies</td>
<td>450, 516</td>
</tr>
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<td></td>
<td>Placenta</td>
<td>Syncytiotrophoblasts</td>
<td>Maternal IgG</td>
<td>A-BL</td>
<td>FcRn Maternal Tf receptor via unidentified carrier</td>
<td>Apical location of receptor and HFE receptor modulator; ferroportin and endogenous copper oxidase believed to facilitate iron release at basolateral PM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Iron</td>
<td>BL A</td>
<td>Tf receptor and FcRn via unidentified carriers</td>
<td></td>
<td>14</td>
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TABLE 1—Continued

<table>
<thead>
<tr>
<th>Organ System</th>
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<th>Cargo</th>
<th>Direction</th>
<th>Receptor/Carrier</th>
<th>Comments</th>
<th>Reference No.</th>
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</thead>
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<tr>
<td>Lung</td>
<td>Trachea</td>
<td>HRP, ferritin</td>
<td>A-BL</td>
<td>Unknown</td>
<td>Presence in large endosomes; degradation also?</td>
<td>469</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A-BL at low levels may be entry point for pathogens</td>
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<td>Upper airways</td>
<td>IgA</td>
<td>BL-A</td>
<td>IgA receptor via CCV</td>
<td>Degraded fragments released at BL (Ussing chamber)</td>
<td>273</td>
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<td>Bronchial epithelium</td>
<td>Albumin</td>
<td>A-BL</td>
<td>Unknown</td>
<td>Transport of intact protein determined by bioassay</td>
<td>269</td>
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<td>Bioactive-Fc fusion protein</td>
<td>A-BL</td>
<td>FcRn</td>
<td></td>
<td>553</td>
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<td>Perfused rat lung</td>
<td>Albumin</td>
<td>A-BL</td>
<td>g60 in caveolae</td>
<td>Question of which alveolar cell type involved</td>
<td>267</td>
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<tr>
<td>Mammary gland</td>
<td>Alveolar epithelium</td>
<td>dlgA</td>
<td>BL-A</td>
<td>IgA-receptor</td>
<td>Presumed to be same as in intestine and liver</td>
<td>497</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Transferrin synthesized by lactating gland; pathway of iron unknown</td>
<td>310</td>
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<tr>
<td></td>
<td></td>
<td>IgG</td>
<td>BL-A</td>
<td>Tf receptor via unidentified carrier</td>
<td>Neonatal rodent only</td>
<td>245</td>
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<td>Thyroid epithelial cells</td>
<td>Thyroglobulin</td>
<td>A-BL</td>
<td>Megalin-mediated endocytosis via CCV</td>
<td>Separate route for Thy-T&lt;sub&gt;3&lt;/sub&gt;/T&lt;sub&gt;4&lt;/sub&gt; (lysosomes)</td>
<td>352</td>
</tr>
</tbody>
</table>

A, apical; BL, basolateral; HFE, hemachromatosis gene product; PM, plasma membrane; Tf, transferrin; TCII, transcobalamin II; CCV, clathrin-coated vesicle; Thy, thyroglobulin; T<sub>3</sub>/T<sub>4</sub>, thyroxines; pIgA, polymeric IgA; FcRn, IgG receptor; LDL, low-density lipoprotein.

Fig. 1. The ultrastructure of a capillary network, an endothelial cell, its membrane, and caveolae. A: the vascular casts of the forestomach are shown in this scanning electron micrograph. The submucosal vessels are seen under the two-dimensional mucosal capillary network. [From Imada et al. (254), copyright 1987 Springer-Verlag.] B: this transmission electron micrograph is representative of the ultrastructure of an endothelial cell if the capillaries indicated in A were viewed in cross section. [From Bolender (40) by copyright permission of The Rockefeller University Press.] C: a higher magnification of the indicated region of the endothelial cell in B. The caveolae attached to both the luminal and basal plasma membrane (PM) domains are indicated. [From Fawcett (147), with permission from Journal of Histochemistry & Cytochemistry.] D: a higher magnification of the indicated caveolae in C. Caveolae (vesicles; v) open to the blood or tissue fronts or that appear to be completely closed are indicated. [From Bruns and Palade (65) by copyright permission of The Rockefeller University Press.]
controversy between physiologists and morphologists for over 50 years. Numerous reviews documenting the history, experimental details, and different interpretations have appeared in this and other journals (377, 467, 539, 571, 608, 614). Because the controversy centers on whether tight junctions or caveolae serve as the major (only) conduit for transported cargo, we will briefly recap this story.

On the basis of experiments in which he compared the compositions of the blood and lymph in the hindleg muscle of cats injected with various size tracers, Pappenheimer et al. (428) postulated in 1951 that plasma components (ions, small solutes, and proteins) were transported through two types of rigid pores: a small, ~3- to 5-nm-diameter pore present at a frequency of 100/μm² and a large, ~20- to 40-nm-diameter pore present at 1% the frequency of the small ones (428). When the endothelium was seen at the ultrastructural level, no structures corresponding to the postulated pores were found. Instead, caveolae were observed, leading Bruns and Palade (65, 66) to suggest that they performed the function ascribed to the rigid pores. In the early years of this controversy, differences in the cell systems, approaches, and tracers used by researchers in the two camps often yielded conflicting results with differences in interpretations. However, both sides progressively refined their experimental approaches and have arrived at an apparent consensus: caveolae do play a role in transport across endothelia, either as fused channels (physiologists) or bona fide transport vesicles (cell biologists). Our position is that caveolae contribute to the high permeability of continuous endothelia. However, because their number exceeds the number of functional pores predicted by Pappenheimer’s results, there must be other functions for caveolae. In fact, they have been proposed to harbor signal transduction components in both active and inactive states (9, 546). The recent reports of mice genetically engineered to lack the protein caveolin-1, a major component of caveolae, are particularly relevant and are discussed in section iv.

Although the controversy has focused on bulk-phase transcytosis, receptor-mediated transcytosis of specific macromolecules also takes place across the endothelia of the microvasculature (Table 1). Albumin and orosomucoid are both transcytosed in comparable, saturable, and temperature-dependent fashions, and caveolae mediate their transport. A putative receptor for albumin of ~60 kDa that is present only on continuous capillary endothelia has been identified by several groups (Table 1), but it has not yet been cloned and sequenced. The transcytosis of IgG across continuous endothelial cells is particularly interesting, in light of the expression of the neonatal Fc receptor (FcRn) by these cells (47) and the receptor’s role in maintaining high serum IgG in the adult (Table 1). Does the receptor work in both the apical-to-basal and reverse directions? Are clathrin-coated vesicles used, as they are to carry maternal IgG from the gut to the interstitium (apical to basolateral) in neonatal rodents (Table 1)? Is excess IgG degraded when the endothelial FcRn receptor is saturated with its ligand, absent, or dysfunctional? If so, how? This area of endothelial cell biology deserves further study, because it might reveal the mechanism(s) used to selectively deliver a ligand (IgG) from the interstitium back to the circulation (basolateral to apical). We will return to several of these issues below. Finally, one polypeptide hormone, human luteinizing hormone/chorionic gonadotrophin (hLH/CG), is reportedly transcytosed via clathrin-coated pits and vesicles across the continuous endothelium of the testis (Table 1). The transport is apparently mediated by the same receptor present on Leydig cells, the target of the hormone in the testis. This system deserves further study, because it is one of two documented examples in which clathrin-coated vesicles of an endothelium transcytose cargo; the other is brain endothelia and transferrin-Fe (see sect. iiB1c).

B. Transcytosis in the Brain

Since the 19th century dye experiments of Ehrlich, the brain has been known as a “privileged” organ where access is tightly regulated so that the environment remains chemically stable. The brain’s fluid is different from either the blood or noncerebral tissue. The two principal gatekeepers of the brain are the cerebral capillary endothelium and the epithelial cells of the choroid plexus (Fig. 2A). These cellular barriers are specialized for the passage of different nutrients from the blood (132, 552). The capillaries move nutrients that are required rapidly and in large quantities, such as glucose and amino acids. These small molecules are transported by membrane carriers using facilitated diffusion. The choroid plexus supplies nutrients that are required less acutely and in lower quantities. These are folate and other vitamins, ascorbate, and deoxyribonucleotides. Their transport requires energy since the blood concentrations of these nutrients are extremely low. Of relevance to this review is experimental evidence that transcytosis of a limited set of macromolecules occurs across brain capillaries from blood to the interstitium (the blood-brain barrier) but does not occur across the epithelial cells of the choroid plexus into the cerebrospinal fluid [the blood-cerebrospinal fluid (CSF) barrier].

1. Cerebral capillaries

Compared with other organs, the abundance of capillary endothelium in brain is very high (35). At the same time, permeability is about two orders of magnitude lower than that of endothelia in peripheral organs, giving rise to the designation of this endothelium as “tight continuous” (201).
Two features of brain endothelia are different from endothelia in the periphery. Brain endothelial cells have the lowest frequency of caveolae (<100/µm²), and the character of their tight junctions is influenced by underlying astrocytes through the actions of soluble factors, including cytokines (111, 263). Claudins 1 and 5 as well as occludin appear to be relevant players in providing a particularly tight junction (229, 289, 319, 385). Very little macromolecular cargo is transcytosed across the cerebral capillary endothelium. The three best-studied ligands are insulin, LDL-cholesterol, and iron; questions and controversy surround each.

A) INSULIN. The finding that insulin-sensitive glucose transporters (GLUT 4) were present in the brain (312, 369) led to the search for insulin receptors on endothelial cells in the brain, and it was anticipated that insulin receptors would also be present in the cerebral capillary endothelium. However, insulin receptors were never detected in the cerebral capillary endothelium. Instead, insulin-sensitive glucose transporters are provided by insulin-sensitive astrocytes that extend processes into the brain capillary wall where they are surrounded by endothelial cells. These astrocyte-intercalated cerebral capillaries have a very high density of astrocytic processes that extend between the cerebral capillary endothelial cells and that provide insulin-sensitive glucose transporters to the exchange zone between blood and brain. Therefore, insulin-sensitive glucose transporters are present in these astrocyte-intercalated cerebral capillaries, and insulin receptors are absent from these cerebral capillary endothelial cells.
Iron and hormonal effects on the cells. Although receptors were found (431, 592), the current status of insulin’s transport by brain capillaries is not resolved.

**B) LDL.** Cells in the brain require cholesterol, which is synthesized endogenously (127), but can also be provided by the transcytosis of plasma LDL intact across brain endothelium (112). There is good evidence for the presence of LDL receptors on the luminal PM of cerebral endothelium (376). Such expression is unusual, since cells that are constantly exposed to the high LDL levels in plasma normally downregulate their LDL receptors. Experimental evidence from in vitro studies indicates that cholesterol levels in the underlying astrocytes play a role in regulating LDL receptor expression levels in the overlying endothelial cells (111). The puzzle here is how a cell distinguishes between LDL for its own needs and LDL for use by cells behind the permeability barrier it forms. In the periphery, this seems to have been solved by receptor-mediated endocytosis via clathrin-coated pits/vesicles for internal use versus fluid-phase (non-receptor-mediated) transcytosis via caveolae for use by interstitial cells (600). However, in the brain, there is virtually no fluid-phase (i.e., nonselective) transcytosis. Thus it will be important to localize LDL receptors in brain endothelium at the ultrastructural level; are they in caveolae or clathrin-coated pits? Another important issue is how transcytosed cholesterol is presented at the abluminal surface of endothelial cells, since apoprotein B, which is the apoprotein-carrying cholesterol in the circulation, is not present in CSF. Apoproteins A1 and E are the principal cholesterol-carrying molecules in the brain (127, 629). Underlying pericytes of the brain endothelium have been characterized as phagocytic; perhaps they participate in the degradation of apoprotein B and release of cholesterol into the CSF. Cholesterol dynamics in the brain have been reviewed recently (624).

**C) Iron.** Iron is also transported across the blood-brain barrier, but there is conflicting data as to whether it is delivered with or without transferrin (Tf), the principal iron-carrying protein of plasma (52). (Iron and Tf are discussed in more detail in sect. uD2.) While several in vivo studies have reported that injected $^{59}$Fe-Tf does not accumulate to the same extent as $^{59}$Fe administered similarily (104), others report equivalent accumulations (152, 635). Tf receptors are definitely present on brain endothelium (248), and Tf is internalized by brain endothelium in vivo via clathrin-coated vesicles (476), leading some to speculate that plasma Tf may be carrying Fe across and then recycling back unloaded (52, 384, 429, 430, 545, 591, 635). Such a scenario would require a milieu on the basal side of sufficiently low pH to effect iron’s release. Because the pH of the underlying interstitium in brain is not known to be acidic, there must be novel dissociation mechanisms not yet discovered. Whatever the mechanism, iron is not free in the brain interstitium but is complexed to Tf. Again, there is conflicting data about the source of this protein. Tf is synthesized and secreted by the epithelial cells of the choroid plexus (384). However, hypotransferrinemic mice have been shown to accumulate substantial amounts of intraperitoneally administered human transferrin intracranially, indicating that the endogenous source could also be derived from the serum (126).

The brain endothelial insulin and Tf-Fe transport systems have received attention from researchers working on therapeutic drug delivery systems (96, 161, 165). An anti-Tf-receptor antibody, OX26, is transcytosed into the brain mass, but the amounts are extremely low, <1% of the antibody injected. Although this amount may be sufficient for drug delivery, it is not definitive evidence for quantitative transcytosis of the receptor along with its cargo. Nonetheless, this approach is being combined with toxins that bind to specific claudins and transiently open tight junctions, to deliver macromolecular drugs (100).

**D) Immunoglobulin G.** It turns out that brain endothelial cells express the FcRn and transport intracranially delivered IgG out of the brain in a receptor-mediated fashion (Table 1). The question is how circulating IgG initially crosses into the brain. As for the peripheral endothelium, the vesicular carrier and molecular mechanisms responsible for IgG transport are as yet unknown.

### 2. Choroid plexus

The choroid plexus is composed of a highly convoluted sheet of cuboidal ependymal epithelium that sits on a closely apposed basal lamina. Both morphological and biochemical tracers have provided good experimental evidence that the apical tight junctions of these epithelial cells are the blood-CSF barrier in the choroid plexus (see Fig. 2A). To date, no obvious ultrastructural or molecular features distinguish these junctions from neighboring ependymal cells, but we would predict that specific claudins are responsible for this difference (323). Interestingly, the basal lamina may act as an inducer of the tight junction specializations that make this cell type highly impermeable to macromolecules (reviewed in Ref. 590). The epithelial cells make much of the CSF that nourishes and cushions the brain. The protein content of CSF (25 mg/100 ml) is low relative to that of plasma (~6,500 mg/100 ml), and the composition is different. Transthyretin, which binds thyroxine, and Tf are made and secreted by the epithelial cells, while apoproteins E and A1, which are present in lipoprotein particles in the CSF, are made by astrocytes. The presence of these proteins in CSF raises the obvious questions of where and how their ligands, presumably from the blood, reach them? Furthermore, are the components of CSF fluid functionally accessible to brain tissue or part of a drainage system much...
like the lymphatics? To our knowledge, there are no definitive answers.

What about transcytosis in the choroid plexus? Interestingly, although endocytosis is robust at the basal surface of the epithelial cells, transcytosis across to the apical environment is minimal to nonexistent; rather, virtually all tracers internalized from the basal side end up in lysosomes (590). The endocytic activity may reflect the high permeability of the fenestrated capillaries that supply the choroid plexus and hence the abundance of plasma proteins bathing the basal side of these cells. Van Deurs (589) examined transcytosis in the apical to basolateral direction as a possible route for elimination of waste from the CSF. Intraventricular injection of soluble horseradish peroxidase and cationized ferritin resulted in their overwhelming delivery to lysosomes; very small amounts appeared in coated pits along the lateral surface (589). The conclusion that apical-to-basolateral transcytosis was not an active pathway has been confirmed by others (24) using additional electron microscopic (EM) tracers.

C. Immunological Protection and Transcytosis

At several stages in the intricate choreography of the vertebrate immune response, transcytosis is used to move antigens and protective antibodies across epithelial barriers (Table 1). “Antigen sampling” is the first step in the mucosal immune response and entails the apical-to-basolateral delivery of soluble and particulate antigens to underlying mucosal-associated lymphoid tissue. This transcytotic event is carried out principally by M cells that are located in lymphoid follicle-associated epithelium throughout the gastrointestinal and urogenital tracts (175, 401, 402, 579, 621). Later in the mucosal immune response, polymeric IgA, secreted by appropriately activated plasma cells, is transcytosed along the basolateral-to-apical axis by epithelial cells in the digestive tract, liver, and mammary gland and is released as secretory IgA into the gut lumen, bile, and milk, respectively (245, 302, 393). The third use of transcytosis occurs in a form of systemic immune protection, termed “passive immunity,” which is the transport of maternal IgG to the developing fetus or neonate. Species differences dictate whether maternal blood or milk is the source of the IgG and whether the placenta or the intestine is the site of this transfer (178, 245). Certainly, the last two transcytotic processes start with uptake of their cargo through clathrin-coated pits/vesicles and may transit through parts of the endosomal system. Thus the mechanisms regulating these itineraries most likely differ from those used by endothelial cells, where a caveolar pathway predominates.

1. Structural features of intestinal epithelial cells

Figure 3 shows the tissue organization and ultrastructural appearances of M cells and enterocytes (adhesive columnar cells), the two epithelial cells participating in transcytosis in the intestine. These cells are very different from one another and the capillary endothelial cell. Depending on the species, M cells comprise a variable but small percentage of the epithelia overlying organized mucosal-associated lymphoid tissue, making them a very minor cell population in the gastrointestinal tract. Being epithelial cells, their basal extensions sit on a basal lamina, but much of their basal membrane lines an extracellular “pocket” in which migrating monocytes and lymphocytes accumulate. As can be seen in Figure 3B, the pocket is a short distance from the apical surface. Thus these cells have evolved a short transcellular pathway much like the endothelial cells, but in contrast they have few to no caveolae; rather, coated pits are present on the apical PM. Figure 3B also shows that M cells do not have the luxuriant brush border that is present on adjacent absorptive enterocytes. They have short microvilli, or microvilli, hence the name M cells. In contrast, absorptive enterocytes are simple columnar cells with several apical features in addition to their brush borders (Fig. 3C). Clathrin-coated pits are present at the base of microvilli, and a thick glyocalyx composed of integral membrane proteins with glycosaminoglycan side chains emanates from the microvillar membrane. This latter structural feature as well as the rigidity of the microvilli are thought to prohibit microorganisms from attaching and invading enterocytes. The intracellular organization of these columnar epithelial cells is also polarized, with basally located nuclei, supranuclear Golgi, and an abundance of pleiomorphic membrane compartments underlying the terminal web of the brush border (Fig. 3C). The basolateral-to-apical length of this cell is ~20 versus 0.2 μm for a capillary endothelial cell, making the transcytotic route across enterocytes potentially much longer. Furthermore, microtubules are an important structural element of the transcytotic pathway in enterocytes, but not in M or endothelial cells.

2. M cells, transcytosis, and antigen sampling

Quite early, researchers studying the routes of pathogen invasion discerned that specific regions of the intestine collected adherent particulate material present in the gut lumen; when the regions were visualized at the EM level, M cells were identified as the invasion route (72, 579, 621). The transcytotic route across M cells is thought to be part of the mechanism by which antigens are routinely sampled along the entire mucosal surface. Not surprisingly, numerous pathogens have evolved mechanisms to exploit the transcytotic process as a means to invade and disseminate before a strong enough immune re-

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A response can be mounted (403, 464). In recent years, this route of entry has been studied intensively in the hopes of understanding the basic mechanisms of antigen sampling and developing effective vaccine delivery systems against stealthy invaders. Because adherence is an essential first step in invasion, researchers have focused on identifying the molecular basis for the selective adherence of antigens and pathogens to M cells and not adjacent enterocytes. Lectin staining in situ has been used in attempts to identify particular glycosidic moieties that might be differentially expressed by M cells (160, 186, 187). Recently, β1-integrin was localized to the M cell apical surface and proposed as the receptor for several pathogens (257–259). This membrane protein, which is expressed on the basolateral surface of neighboring absorptive cells, has a cytoplasmic tail that could mediate the endocytosis of particles bound to its extracellular domain. In support of this notion, M cells are avidly endocytic and their apical membrane is much more dynamic than the rigid and stable brush border of the enterocyte. In fact, both phagocytic and pinocytic mechanisms appear to operate at the apical surface of these cells. Adsorbed macromolecules are en-

**FIG. 3.** The small intestine (ileum) contains Peyer’s patches. A: a schematic drawing of a Peyer’s patch is shown that illustrates the general arrangement of gut-associated lymphoid tissue. Lymphoid follicles in the submucosa are associated with dome areas that extend into the gut lumen. The domes are covered with specialized epithelium that contains M cells. B lymphocytes mainly populate the lymphoid follicles, while T cells predominate the interfollicular areas. High endothelial venules (HEV) in the interfollicular areas are the route through which lymphocytes enter the Peyer’s patch. [From Gebert et al. (175) by copyright permission of Academic Press.] B: a transmission electron micrograph of an M cell and adjacent columnar cells (CC) from the region indicated in A. The typical M cell has short, irregular microvilli and a basolateral pocket (P) into which the lymphoid cells (here resembling plasma cells) and macrophages migrate. Luminal antigens are endocytosed, transported across the apical cytoplasm (bracket), and delivered to the basolateral pocket. [From Weltzin et al. (616) by copyright permission of The Rockefeller University Press.] C: a low-magnification transmission electron micrograph of several absorptive cells and part of a goblet cell from the region indicated in A is shown from a fasted rat. The lumen of the intestine is at the top and a small portion of the lamina propria (LP) is shown at the bottom. A thin basement membrane separates the basal surfaces (BL) of the cells from the lamina propria. An elongated nucleus is located in the basal region of the cell under which is a dense cluster of mitochondria and few free ribosomes and rough endoplasmic reticulum (RER). The apical cytoplasm contains long mitochondria, a prominent Golgi component (G), RER, and smooth endoplasmic reticulum (SER) concentrated at the terminal web (TW). The free surface is covered by microvilli (Mv). L, lipid droplet. [From Cardell et al. (77) by copyright permission of The Rockefeller University Press.]

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docytosed via clathrin-coated vesicles (404) and delivered to a prelysosomal/lysosomal compartment from which they are released into the underlying pocket for subsequent uptake by lymphocytes and macrophages (404, 425, 616). Thus, unlike endothelial cell transcytosis, lysosomes appear to play a role in M cell transcytosis. Whether the cargo in this compartment is modified by acid hydrolases present in it (6) is not currently known.

3. Transcytosis of IgA

The large amount of mucosa-associated lymphoid tissue and its specialization for the production of IgA make IgA the major immunoglobulin in humans (301, 389). Given that it is synthesized and secreted by plasma cells located in the lamina propria of the digestive, respiratory, and urogenital tracts yet functions in external secretions, IgA must be delivered across an epithelial barrier. This requirement is accomplished by the polymeric IgA receptor (pIgA-R), a single transmembrane protein synthesized by the epithelial cells. As discussed in detail in section IV, this receptor has a long (~100 amino acid) cytoplasmic tail that contains most of the signals necessary to direct it through its cellular itinerary. However, unlike most other endocytic receptors that perform repeated rounds of cargo uptake, delivery and recycling, the extracellular domain of pIgA-R is cleaved upon delivery to the apical surface and released into the lumen with its ligand. The presence of the added “secretory component” stabilizes IgA in the gut lumen. This unique transcytotic system is expressed in many epithelia throughout the body, including kidney, trachea, and the digestive tract, including the liver (Table 1). Interestingly, some pathogens appear to have exploited the small percentage of uncleaved plgA-R present in the apical membrane of nasopharyngeal epithelial cells to gain entry into the underlying interstitium (Table 1). This result suggests that the receptor is able or can be coerced to transcytose in an apical-to-basolateral direction. The mechanism, whether normal or pathogen induced, may have therapeutic potential.

4. Transcytosis of IgG

The transfer of maternal immunoglobulins to fetal or neonatal offspring provides the latter with systemic immunity until their immune system matures. Several organs transport IgG-type immunoglobulins (245). As with IgA, maternal IgG must be transcytosed across an epithelial barrier. In all mammalian species, it is transcytosed in an apical-to-basal direction. Thus in humans, IgG in the maternal blood is transported across the placenta (Fig. 2B), while in rodents, maternal IgG is first delivered into milk, a basal-to-apical route, and secondarily across the absorptive epithelial cells of the small intestine, an apical-to-basolateral route.

The receptor mediating the apical-to-basal transport of IgG is FcRn, a distant member of the major histocompatibility complex (MHC) I family (542). As for other MHC I proteins, the FcRn is a heterodimer, with a transmembrane heavy chain and β2-microglobulin light chain. The heavy chain has a cytoplasmic tail containing an internalization motif that mediates endocytosis of maternal IgG via clathrin-coated pits and vesicles present at the base of the apical brush border of neonatal rodent enterocytes. The receptor and ligand are transported through an endosomal compartment to the lateral surface of these cells (1). Thus this transport system shares the property with M cells of using a prelysosomal compartment to deliver its cargo. However, the unique pH dependence of binding allows the ligand to remain associated with the receptor at low pH (in the gut lumen and through slightly acidic endosomes) and be released at neutral pH (in the interstitial space) without apparent modification. The FcRn recycles back to the apical PM in neonatal intestine.

Although the finding that β2-microglobulin (β2-M) knock-out mice lacked the apical-to-basal IgG transport system in the neonatal intestine was expected, it was initially surprising that circulating IgG in β2-M-null adults exhibited a much shorter half-life than in wild-type mice (177). This result suggested that FcRn played a role in IgG homeostasis (541), confirming a long-standing hypothesis by Brambell (54) that the prolonged circulation of IgG in plasma was due to a receptor capable of protecting IgG from degradation. Given the pH dependence of IgG binding to FcRn, the current view is that intracellular FcRn binds nonspecifically endocytosed IgG within an endosome-like compartment and returns it to the circulation (177). The tissues and cells performing the protective function may be hepatocytes or endothelial cells, since both express FcRn at the PM. Quantitative studies are needed.

An important finding in the β2-M knock-out mouse studies was that the levels of maternal IgG in colostrum and milk were normal, indicating that FcRn does not play a role in the transcytosis of IgG in the rodent mammary gland. This is not so surprising, considering that the direction of transport is opposite to that in the placenta or intestine, although in endothelial cells, the FcRn presumably carries IgG in the basal-to-apical direction (Table 1). The identity of the mammary gland receptor system will be important to determine.

D. Role for Transcytosis in the Homeostasis of Micronutrients

Most vitamins, essential minerals, and trace elements, collectively called micronutrients, come from the diet. Thus they must cross an epithelial barrier some-
where along the digestive tract; this occurs primarily at the level of the intestine. However, transcytosis is the least used route for micronutrient absorption. Lipid-soluble vitamins associate with bile acid micelles in the gut lumen and are thought to then partition progressively and passively across absorptive cells, associating with chylomicra somewhere before or at the basal side of the cells. Dietary vitamin B\textsubscript{12} (cobalamin) is an exception, because it uses vesicle-mediated steps, in part, to cross intestinal cells. Many minerals are assumed to be absorbed paracellularly (61). This assumption is based on calculations of transit times and absorption rates. However, dietary iron is transported across the intestinal epithelium via multiple membrane transporters; once in the circulation, its delivery to the brain and fetus requires transcytosis. Additionally, Cu and Zn, as well as other heavy metals, appear to be transported into intestinal absorptive cells via membrane transporters at the apical plasma membrane. Finally, the kidney proximal tubule cells provide an important function in vitamin homeostasis by avidly scavenging several vitamins (Table 1) from the urine using a modified type of transcytosis.

1. Vitamin B\textsubscript{12}

All cells require vitamin B\textsubscript{12} as a coenzyme in one-carbon transfers. Methyl malonyl CoA mutase uses it in the adenosyl-B\textsubscript{12} form to convert methyl malonyl CoA to succinyl CoA in the mitochondria; methionine synthetase uses it in the methyl-B\textsubscript{12} form to convert homocysteine to methionine in the cytoplasm. B\textsubscript{12}'s journey to the cytoplasm of all cells is a fascinating and curiously convoluted process. The players so far identified are listed in Table 2 and placed in cellular context in Figure 4A. Several reviews cover this topic in more detail (276, 488, 514).

A) UPTAKE FROM THE INTESTINAL LUMEN. In carnivores, B\textsubscript{12} is present in ingested meat as the cofactors mentioned above. Upon digestion by pancreatic enzymes in the small intestine, free B\textsubscript{12} is bound by intrinsic factor (IF), a 27-kDa glycoprotein secreted by parietal cells of the stomach (317). In the terminal ileum of the small intestine, the luminal B\textsubscript{12}-IF complex binds to its receptor, cubulin, a large membrane-associated glycoprotein that is located in the microvillar brush border of absorptive enterocytes (515). Cubulin’s association with megalin, a member of the LDL receptor-related (LRP) family of endocytic receptors (reviewed in Refs. 192, 619), leads to the internalization via clathrin-coated vesicles of the entire cubulin-IF-B\textsubscript{12} complex. After delivery of the IF-B\textsubscript{12} complex to endosomes, cubulin and megalin recycle for further rounds of endocytosis; since cubulin’s association with megalin is stable at pH 5, it is thought to stay associated throughout. Meanwhile, dissociated B\textsubscript{12}-IF is delivered to lysosomes, where the protein is degraded by leupeptin-inhibitable acid hydrolases (196) and B\textsubscript{12} is transported out of lysosomes. This last step is mediated by a yet-to-be-discovered transporter(s). Interestingly, in this pathway there is no avoidance of lysosomes; to the contrary, lysosomal function is essential, since failure to degrade IF within the intestine results in a B\textsubscript{12} deficiency in all subsequent tissues and cells. Some investigators are exploring the B\textsubscript{12} entry pathway as a means to deliver drugs orally (487).

**Table 2. Vitamin B\textsubscript{12} (cobalamin)**

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Site of Synthesis/Expression</th>
<th>Biochemical Characteristics</th>
<th>Functions</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>R binders (haptocorrins)</td>
<td>Salivary gland, stomach, placenta, circulation, and granulocytes</td>
<td>Small secreted glycoproteins (includes TCI, not a glycoprotein)</td>
<td>Bind cobalamin</td>
<td>69</td>
</tr>
<tr>
<td>Intrinsic factor (IF) Cubulin</td>
<td>Parietal cells/stomach Enterocyte in terminal ileum</td>
<td>27-kDa secreted glycoprotein 400-kDa peripheral membrane glycoprotein secreted apically by enterocyte</td>
<td>Binds B\textsubscript{12} in small intestine Binds B\textsubscript{12}-IF complex at apical PM</td>
<td>317 294 515</td>
</tr>
<tr>
<td>Megalin</td>
<td>Enterocyte in terminal ileum; also present in kidney proximal tubule, placenta, thyroid</td>
<td>600-kDa transmembrane glycoprotein, clathrin internalization motif in cytoplasmic tail</td>
<td>Apical PM receptor that binds cubulin-B\textsubscript{12} and TCI-B\textsubscript{12} complexes in Ca-dependent manner and mediates endocytosis; recycles Binds B\textsubscript{12} in circulation</td>
<td>448 460 463</td>
</tr>
<tr>
<td>Transcobalamin binding protein (TCII) TCI and III</td>
<td>Liver and enterocyte Granulocytes Kidney, liver, placenta, intestine</td>
<td>45.5-kDa secreted protein Secreted glycoproteins 62-kDa transmembrane glycoprotein</td>
<td>Bind B\textsubscript{12}; unknown functions Basolateral PM receptor for TCI-B12</td>
<td>49 518</td>
</tr>
</tbody>
</table>

Definitions are as in Table 1.
B) TRANSFER TO CIRCULATING TRANSCOBALAMIN II AND TRANSCYTOSIS. A puzzle is the mechanism by which cytoplasmic B$_{12}$ is subsequently transported into the basal milieu surrounding the enterocyte. Extracellular transcobalamin (TC) II, a 40-kDa protein in the interstitium/circulation, serves as the major functional carrier of B$_{12}$ (Table 2). (TCI and TCIII are also B$_{12}$ carriers, but their functions remain unknown.) TCII is synthesized and secreted primarily by the liver (206, 495). Evidence that enterocytes express a TCII transcript (460) suggests that B$_{12}$ may be loaded onto newly synthesized protein as it transits the secretory pathway (463). The B$_{12}$-TCII complex would then be released at the basolateral surface of the enterocyte. Of course, this scenario requires the presence of a B$_{12}$ membrane transporter in the secretory pathway.

Once in the circulation, how does TCII-B$_{12}$ reach cells? All cells express a TCII receptor, which mediates the endocytosis of the complex via a clathrin-mediated mechanism. The TCII receptor is a single transmembrane glycoprotein of 62 kDa (49) that functions as a homodimer at the plasma membrane (Table 2). After internalization, the TCII-B$_{12}$ complex is delivered to lysosomes, where TCII is degraded and B$_{12}$ is again transported into the cytoplasm for subsequent use as a
cofactor (249). But how does B12 reach cells behind a selective barrier, for example, the brain or testis? Although a B12-TCII receptor on brain or testicular endothelial cells has not been reported, we predict that it must be there. Could it be the same receptor as that found on the basolateral PM of most epithelial cells, even though it would be on the apical PM of these endothelia? What is the mode of transcytosis and the subsequent fate of the TCII protein and B12 in brain endothelial cells? Are the fates different from those in other cells? Clearly, this system would be interesting to explore further.

C) INVOLVEMENT OF ADDITIONAL EPITHELIA IN B12 HOMEOSTASIS.

There are additional aspects of B12 homeostasis that deserve comment. The kidney, yolk sac, and placenta express the protein components involved in intestinal B12 absorption (294). For example, cubilin is very abundant in the kidney proximal tubules, where it can bind and internalize B12-IF, again via megalin (91, 517). This is strange, since IF is not normally found in the circulation. Not unexpectedly, given the small size of TCII and thus its filtration by the glomerulus, there is a scavenger of B12-TCII in the kidney, and it is megalin itself on the apical PM of proximal tubule cells. Similar to its behavior in the intestine, megalin internalizes the protein-B12 complex and delivers it to lysosomes, where TCII is degraded and B12 is stored in a form that is retrievable into the circulation upon demand. This last step, storage of B12 points to possible differences between B12 dynamics in intestinal and kidney epithelial cells and is worth following up. Finally, antibodies to cubilin cause severe defects in developing fetuses, possibly due to a failure to deliver B12 and/or other essential components to the developing central nervous system via the yolk sac (516). As can be seen in Table 1, the cubilin-megalin system has the capacity to bind and move many different cargo molecules.

2. Iron

An essential cofactor in the homeostasis of every cell, iron in mammals exists in three predominant forms: bound to the circulating plasma protein transferrin; as heme in intracellular proteins such as mitochondrial cytochromes, hemoglobin, and myoglobin; and in ferritin, the storage form of iron (5). Iron is avidly reutilized by mammals, and thus the daily requirement for it is small. Of 3–4 g total body iron, only 1–2 mg are lost per day through desquamation. However, because there is no mechanism for its disposal, excess iron leads to disease (525, 623). Furthermore, the insolubility of both valence states at neutral pH and the toxicity of Fe2+ in the presence of oxygen makes control of this essential micronutrient especially vital. Studies of both iron deficiency and overload in human disease and animal models have helped to elucidate the mechanisms of iron homeostasis.

There are many excellent reviews covering different aspects of iron metabolism (5, 11, 203, 277, 278, 485).

A) TRANSPORT ACROSS THE INTESTINE APPEARS NOT TO REQUIRE VESICULAR CARRIERS. Dietary iron is initially absorbed in the duodenum in either the heme and nonheme (free or chelated) form; much more is known about uptake of the latter. The current consensus is that free Fe2+ is transported directly across the apical membrane of absorptive epithelial cells via a multistep process (203) involving the divalent metal transporter DMT-1 (Fig. 4B) (202). The protein accepts a broad range of divalent ions. Older reports in the literature implicated a vesicular process for iron’s uptake, consisting of a mucin at the apical surface binding free iron, followed by its import via an integrin and transfer to a molecule termed “mobilferrin,” which was later identified as calreticulin, an endoplasmic reticulum lumen chaperone. With the identification of DMT-1, the “mobilferrin” hypothesis is now open to question (12).

After transport into the epithelial cell, cytoplasmic Fe2+ is subsequently directed across the intestinal basolateral membrane via another newly identified transporter, called ferroportin (372). In intestine, a membrane form of the ferro-oxidase ceruloplasmin, called hephaestin (607), is thought to aid both in the oxidation of Fe2+ and the loading of Fe3+ onto Tf. How this occurs is still unknown, although Caco-2 cells are reportedly capable of transferring apically derived iron onto apo-Tf in the basolateral medium (342). Interestingly, the livers of “atransferrinemic” mice become iron overloaded, implying that a non-Tf-bound transport mechanism must operate to deliver iron from the intestine into the hepatocyte. Possible carriers have recently been reported (reviewed in Ref. 277).

B) UPTAKE OF TF-BOUND IRON. Once in the circulation, iron is carried principally by hepatocyte-derived plasma Tf. Cells directly accessible to the circulation take up iron via the well-studied process of Tf receptor-mediated endocytosis (Fig. 4B); that is, iron bound to Tf is internalized in clathrin-coated vesicles, dissociated from Tf by the low pH in endosomes, and transported across the membrane, most likely by a DMT-like transporter. Apo-Tf bound to its receptor recycles to the same cell surface for subsequent rounds of uptake, delivery, and recycling.

How do cells located behind a selective barrier obtain this essential mineral? And how do cells responsible for transcytosing it obtain sufficient iron for their own metabolic needs? In the adult, iron reaches cells in peripheral tissues by nonselective caveolar-mediated transcytosis of Tf-bound iron across the endothelium; there is no dissociation in transit.

C) TRANSCYTOSIS OF IRON IN THE PLACENTA AND BRAIN. As described in section 4B1 for brain, transcytosis of iron across cerebral capillaries is receptor mediated. Also, as described earlier, the extent to which plasma-derived Tf moves across brain endothelial cells is controversial and presently unresolved (52). There is a similar uncertainty...
in the transcytosis of iron across the human placenta. As for brain endothelium, the direction across the syncytiotrophoblasts is apical to basolateral (Fig. 2A). Is maternal or fetal Tf the carrier? Finally, the fetal endothelial capillary is an additional cellular barrier that must be crossed and the mechanism is currently unresolved.

E. Additional Transcytosis Systems

1. Lung

Transcytosis occurs in the upper regions of the respiratory tract and involves two receptor systems already described, plgA-R and FcRn (Table 1). Secretory IgA is a known constituent of the lung’s immune defense system, with bronchial epithelial cells carrying out basolateral-to-apical transport of dIgA, which is secreted by local plasma cells in underlying lymphoid tissue (reviewed in Refs. 444, 491). A recent study using a clever biological read-out has documented the efficient apical-to-basolateral transcytosis of intact IgG across bronchial epithelium via the FcRn (553). This latter pathway could possibly be exploited to deliver genes systemically. Finally, albumin, which is found in lung fluid, is endocytosed specifically at the apical surface of airway epithelia but is then subsequently degraded. At the alveolar level, the question of whether albumin is transcytosed intact is uncertain (see Ref. 437 for review). Malik and colleagues (267) have recently reported the presence and function in type II epithelial cells of a gp60 membrane protein related to that found in endothelial cells.

2. Mammary gland

Milk is composed largely of locally synthesized nutrients that are secreted by alveolar epithelial cells in the lactating gland (523). However, a substantial fraction of milk proteins is thought to be derived from the serum, meaning that transcytosis must be used to deliver these “exogenous” molecules. To date, distinguishing between the two sources has not been systematically done. Local plasma cells secrete IgA, which is transcytosed by luminal epithelial cells using the plgA-R (Table 1). As already described, rodents but not humans transcytose maternal IgG into milk for several days after pups are born. The receptor and pathway are unknown. Micronutrients in milk are supplied from the maternal circulation, but neither their transcellular path nor the source of binding protein (e.g., for iron, B12, or vitamin D) is clear. Iron is transcytosed, but milk transferrin is synthesized in the gland, potentially necessitating an intracellular transfer. Ca\(^{2+}\) is also derived from the maternal circulation, but its concentration in milk is ~100-fold higher than that in serum. The Golgi Ca\(^{2+}\)-ATPase has been proposed as the pump that sequesters Ca\(^{2+}\) in vesicles, which are subsequently delivered to the apical membrane with release of Ca\(^{2+}\) via exocytosis (523).

3. Thyroid

The thyroid hormones triiodothyronine (T\(_3\)) and thyroxine (T\(_4\)) are produced from their iodinated precursor protein, thyroglobulin, which is stored in the lumen of a thyroid follicle. Upon stimulation by thyroid stimulating hormone at the basolateral surface, apical endocytosis increases and thyroglobulin is internalized. The mechanism of uptake is not yet known and may be nonspecific (143, 226, 483). Endocytic vesicles fuse with lysosomes, and the cathepsins act on thyroglobulin to release 20-kDa fragments containing the hormonogenic regions, which are further cleaved to T\(_3\) and T\(_4\) by endo- and exopeptidases. It is a mystery how the hormones reach the circulation, since T\(_3\) receptors are present in the thyroid cell, yet net movement of the hormones is toward the basolateral surface. Once in the circulation, T\(_3\) and T\(_4\) are bound to their protein carrier, transthyretin, which is synthesized in the liver. The complex is transcytosed across continuous endothelium via caveolae. It is presently unclear how these hormones reach the brain. Perhaps the choroid plexus, which synthesizes transthyretin, plays a role.

Approximately 10% of the thyroglobulin protein internalized from the thyroid follicular lumen is not processed in lysosomes, but is transcytosed intact into the circulation. In the 1980s, Herzog (227) found that this amount did not represent spillover from saturation of a putative lysosomal delivery system, since lysosomes continued to fill as increasing amounts of thyroglobulin were internalized. More recently, researchers have identified megalin as the apical membrane receptor mediating thyroglobulin’s apical-to-basolateral transcytosis across the follicular cell (348, 350, 351). This finding raises several questions. Does megalin also cross the cell and reach the basolateral PM? How does megalin’s cargo avoid lysosomes in the thyroid, since in the intestine, its cargo, vitamin B\(_{12}\), bound to intrinsic factor, is delivered to lysosomes for degradation? The in vitro cell system models described in section III could be useful for answering these questions.

F. Role of Transcytosis in Plasma Membrane Biogenesis In Vivo

Of the in vivo transcytosis systems described so far, a common feature is that the cargo is exogenous. However, in two epithelial cell types, the hepatocyte and the absorptive enterocyte of the intestinal villus, endogenous membrane proteins are themselves the cargo! These cells use basolateral-to-apical transcytosis exclusively (hepatocytes) or in part (enterocytes) as a pathway for the deliv-
ery of specific classes of newly synthesized apical plasma membrane proteins. In hepatocytes we determined some years ago that three single transmembrane domain (TMD) apical proteins and one glycosyl-phosphatidylinositol (GPI)-anchored apical protein followed this route (32, 499). Similar observations were made by Maroux and colleagues (148, 355) in the rabbit intestine for aminopeptidase N. Thus the question is whether such an "indirect" pathway of apical PM biogenesis exists in other epithelia in vivo. Unfortunately, we may never have a definite answer, largely because other epithelial tissues are not sufficiently homogeneous nor as amenable as the liver and intestine to the biochemical approaches that were used. In these studies, animals were first administered radiolabeled amino acids in a "pulse-chase" fashion, then enriched populations of apical and basolateral membranes were isolated, the specific membrane proteins immunoprecipitated and their radioactive content determined. Because hepatocytes constitute >70% of the total cells in the liver, subcellular fractionation methods could be used that yielded preparations of highly enriched hepatocyte organelles. Similarly, intestinal mucosa could be scraped from the surface of everted intestines, thus enriching for epithelial cells and allowing subsequent subcellular fractionation of membranes into apical- and basolateral-enriched fractions.

Why are these in vivo studies important? First, they represent "reality," that is, the physiological situation. Second, the results were different from those reported earlier using in vitro polarized cell models, specifically MDCK cells derived from dog kidney (360, 479). In the latter cells, newly synthesized apical PM proteins were shown to be delivered directly from the trans-Golgi network (TGN) to the apical surface. Gradually, as different epithelial cells and more membrane proteins have been studied, the plasticity in the routes and mechanisms for the delivery and retention of PM proteins in epithelial cells has become apparent. Such a realization reinforces the importance of studying a variety of epithelial cells to learn the full repertoire of mechanisms. It also points to the possibility that transcytosis is an "ancient" route, since all epithelial cells express this transport system.

III. IN VITRO CELL MODELS OF TRANSCYTOSIS

The use of in vitro cell models to study transcytosis has many advantages over in vivo systems. First, variation among animals is eliminated, as is the confounding issue of cargo possibly being modified or endocytosed by cell types other than the one under study. Moreover, in vitro systems can be manipulated in ways not possible in vivo, allowing investigators to measure the effects of different variables (e.g., temperatures, pharmacological agents, etc.) with greater precision and to explore the molecular mechanisms of transcytosis. However, these advantages are offset by the loss of the in vivo context (e.g., cues from extracellular matrix, other cell types), which undoubtedly provides levels of regulation that are missing in vitro. For this reason, it is important to be cautious in extrapolating in vitro results to the in vivo situation and to compare results obtained in the two systems whenever possible.

A. What Constitutes a "Good" Transcytotic Cell Model?

An ideal cell model would faithfully recapitulate the in vivo transcytotic system in the types, amounts, and kinetics of cargo transported across the cellular barrier. If these criteria are met (not a simple feat), we can assume that other parameters, such as cellular organization, relevant machinery, tight junction permeabilities, etc., are in place. What experimental factors should be considered and assessed in establishing a good (i.e., less than ideal) in vitro cell model? Based on a review of the literature and our own experience, we have come up with six issues that pertain to simple (bipolar) epithelia.

An important factor is the choice of substratum. Quite early, physiologists recognized that simple epithelial cells achieved a higher degree of polarity and manifested a more differentiated phenotype when they were grown on porous surfaces with media bathing them on both sides. This three-dimensional arrangement simulates the in vivo condition. Filters of various chemistries and coatings are now commercially available (Table 3). To date, cell growth, morphology, and polarity on different filters have been compared systematically in only a few cases (71, 601).

The issue of pore size is relevant for two reasons. First, cells can migrate through larger pores and grow on the underside of filters (581). Obviously, this situation compares results obtained in the two systems whenever possible.

A third factor to consider in the quest for a good in vitro cell system is the cell seeding density. The goal is to obtain a confluent monolayer of homogeneous cells. For
example, sparse seeding of some cell types can lead to confluence being achieved at different times across the filter. This situation could have a profound influence on the differentiation state of the cells. Caco-2 cells, a well-studied model of intestinal absorptive enterocytes, progressively differentiate over a period of ~15–20 days after achieving confluence. Therefore, they are seeded at high density so that confluence is achieved across the entire filter synchronously. The disadvantage of seeding at high density is the possibility of selecting for more rapidly attaching cells. Likewise, some cell lines alter their phenotype depending on the seeding density. This appears to be the case for placental BeWo cells, which form multiple layers if seeded too high (327). Such a situation could lead to erroneous results and interpretations regarding transcytosis. BeWo cells seeded at low density (subconfluence) will grow into a confluent monolayer (Table 4).

The integrity of the monolayer is obviously vital to every study of transcytosis, and there are different methods for assessing it. Transepithelial electrical resistance (TER) measurements are commonly used as an indication of tight junction integrity in a monolayer, and commercial instruments are available for these measurements (241). For meaningful interpretation of results, especially when studying brain endothelium permeability, it is important to compare the in vivo and in vitro TERs (201). Radiolabeled inulin (molecular mass 5,200 Da), an inert, uncharged molecule that reports paracellular leak, is often used, because it can be added to the apical medium and measured in the opposite bath after various times of incubation. Here, a positive control consisting of parallel measurements made to a filter with no cells is important, since there is not immediate equilibration of a tracer between the two chambers in the absence of cells. A simple method for assessing monolayer integrity is to fill the upper chamber to the top, then leave the cells overnight and measure leak by a fall in the fluid level in the top chamber. The ideal would be to measure the integrity of each filter before using it experimentally, but this is rarely done.

Knowing the extent to which a cell’s surface is polarized at a molecular level is crucial to a meaningful interpretation of the results of transcytotic studies. We have termed this factor the “polarity index,” which is the relative distribution of a membrane component in the two PM domains, apical and basolateral. Since the polarity index of most PM proteins is lower in vitro than in vivo, a larger fraction of a particular transcytotic receptor (and its associated intracellular machinery) may be in the “incorrect” PM domain in vitro. This condition would lead to an apparent higher transcytotic activity in the “wrong” direction. A possible solution would be to determine the in vivo and in vitro polarity indices for the receptor under study and then correct back to the in vivo index.

Finally, we assert that complete “balance sheets” are essential when studying transcytosis in vitro. By this we mean accounting for all of the tracer over the time course of the experiment. Unfortunately, such bookkeeping is not usually done. Of course, two measurements are routinely made: 1) tracer uptake from one side of the monolayer and 2) its appearance on the opposite side. But these two measurements alone are not sufficient for a definitive demonstration of transcytosis. It is also important to determine 3) the intracellular accumulation of the cargo, 4) any possible degradation/metabolism, and 5) the integrity of the cargo that crossed the monolayer. These additional measurements give clues as to whether protein cargo avoided lysosomal proteolysis, a feature of some transcytotic pathways.

It is important here to raise a cautionary note about the nature of tracers used to detect and measure transcytosis. Radiolabeled tracers have often been used in biochemical studies, while macromolecules adsorbed to colloidal gold or coupled to the cytochemical agent, horseradish peroxidase (HRP), have been used in microscopic studies. Because chemical or physical modifications to a

### TABLE 3. Commercially available filters

<table>
<thead>
<tr>
<th>Company</th>
<th>Name</th>
<th>Material</th>
<th>Pore Size, µm</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Costar</td>
<td>Transwell</td>
<td>Polycarbonate</td>
<td>0.1–12</td>
<td>Opaque</td>
</tr>
<tr>
<td></td>
<td>Transwell-Clear</td>
<td>Polyester</td>
<td>0.4–3</td>
<td>Transparent</td>
</tr>
<tr>
<td></td>
<td>Transwell-COL</td>
<td>Polytetrafluorethylene (PTFE)</td>
<td>0.4–3</td>
<td>Coated with bovine collagen (I and II)</td>
</tr>
<tr>
<td>Becton-Dickinson</td>
<td>Falcon</td>
<td>Polytetrafluorethylene (PET) or fluoropolymer (FP)</td>
<td>0.4–8</td>
<td>Transparent, translucent, high and low pore density Coated with bovine collagen (I or IV), laminin, fibronectin, or Matrigel</td>
</tr>
<tr>
<td></td>
<td>Biocat</td>
<td>PET or FP</td>
<td>0.4–8</td>
<td></td>
</tr>
<tr>
<td>Millipore</td>
<td>Millicell-CM(Biopore-CM)</td>
<td>Polystyrene</td>
<td>0.4</td>
<td>Transparent, needs coating</td>
</tr>
<tr>
<td></td>
<td>Millicell-HA</td>
<td>Mixed cellulose esters</td>
<td>0.45</td>
<td>Coating not needed</td>
</tr>
<tr>
<td></td>
<td>Millicell-PCF</td>
<td>Polycarbonate</td>
<td>0.4–12</td>
<td>Coating not needed</td>
</tr>
</tbody>
</table>

[Adapted from Hughson and Hirt (241).]
<table>
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<tr>
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</thead>
<tbody>
<tr>
<td>Peripheral endothelial cells</td>
<td>Bovine pulmonary microvessels</td>
<td>118, 530, 531</td>
<td>0.8-µm pore gelatin-coated polycarbonate filter</td>
<td>11- to 150-kDa proteins</td>
<td>Lung microvessel endothelium less permeable than endothelium of larger vessels in comparative study; 2- to 5-fold higher density of plasmalemmal vesicles in microvessel endothelium</td>
<td>511</td>
</tr>
<tr>
<td>Primary endothelial Bovine pulmonary artery</td>
<td></td>
<td>118</td>
<td>0.4-µm gelatin-coated polycarbonate filter</td>
<td>125I-albumin and HRP</td>
<td>Anti-gp60 antibody stimulated A-BL transcytosis with no change in monolayer integrity</td>
<td>268, 576, 602</td>
</tr>
<tr>
<td>Primary endothelial Bovine pulmonary vein</td>
<td></td>
<td>97</td>
<td>3-µm pore gelatin and fibronectin-coated PET filters; cells used 5–6 days after seeding</td>
<td>Lipoprotein lipase</td>
<td>Intact and active lipase moved BL-A via VLDL receptor/heparin sulfate proteoglycan-specific process</td>
<td>414</td>
</tr>
<tr>
<td>Brain endothelial cells</td>
<td>Cerebral cortex cocultured with endothelial cells on opposite sides of 0.4-µm pore rat tail collagen-coated Millicell CM filter</td>
<td>113–116, 376</td>
<td>TER of 660 after coculture for 7 days; TER maintained &gt; 10 days; expression of γ-glutamyltranspeptidase, P-glycoprotein and alkaline phosphatase; permeability to sucrose, iminid, and propanolol showed good correlation with in vivo; [3H]dextran (70 kDa) used to test integrity of monolayer</td>
<td>LDL</td>
<td>From A-125I-LDL and LDL-Au into multivesicular bodies, but 125I-LDL not degraded, from A-acetylated LDL taken up by another receptor and degraded; concludes that lysosomal pathway is functional in confluent cells. Transferin and iron Lactoferrin</td>
<td>112, 122, 151</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>Collagenase-dissociated human brain cerebral cortex microvessels</td>
<td>560</td>
<td>Collagen-fibronectin-coated Transwell filter</td>
<td>Alzheimer's amyloid beta 1–40 peptide</td>
<td>A-BL preferred; RAGE-mediated in part; no degradation</td>
<td>343</td>
</tr>
<tr>
<td>Intestine</td>
<td>Caco-2 Human primary colon carcinoma</td>
<td>637</td>
<td>Transwell filters; cells used 15–20 days after confluency</td>
<td>BL population of apical PM proteins Endogenous p137 membrane protein Endogenous TCH2 receptor and 125I-TCH2-B12</td>
<td>Single TMD apical proteins transcytossed, but GPI did not Bidirectional transcytosis without degradation A-BL transcytosis preferred</td>
<td>307, 308, 375, 141, 49, 451</td>
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<tr>
<td>Caco-2</td>
<td>TER &gt; 150</td>
<td>Millicell-HA filter</td>
<td>232, but see 8, 400</td>
<td>HIV</td>
<td>S. aureus toxins, enterotoxins A, B and toxic shock syndrome toxin 1 (TSST-1)</td>
<td>Facilitated A-BL movement of toxin B and TSST-1, not toxin A</td>
</tr>
<tr>
<td></td>
<td>TER &gt; 200 14–15 days after seeding cells</td>
<td>Type I collagen coated Millicell HA filter</td>
<td>200</td>
<td>A-BL 4- to 10-fold over BL-A, hydrophobicity index a factor, transport concluded to be adsorptive, not receptor mediated</td>
<td>A-BL conjugate</td>
<td>3-Fold greater A-BL than BL-A of conjugate; ligand not degraded after movement (binding to anti-insulin); transcytosis of free insulin 1/15 of conjugate in A-BL direction; possible oral route for insulin; caution: polarity index of Tfn-R not measured</td>
</tr>
<tr>
<td></td>
<td>In the presence of murine lymphocytes of Peyer's patch Caco-2 cells differentiate into M-like cells</td>
<td>Type I collagen coated Millicell HA filter</td>
<td>284</td>
<td>-6-fold over BL-A, hydrophobicity index a factor, transport concluded to be adsorptive, not receptor mediated</td>
<td>-6-fold over BL-A, hydrophobicity index a factor, transport concluded to be adsorptive, not receptor mediated</td>
<td>-6-fold over BL-A, hydrophobicity index a factor, transport concluded to be adsorptive, not receptor mediated</td>
</tr>
<tr>
<td>T84</td>
<td>Human colon carcinoma</td>
<td>Crypt cell features (ion secretion)</td>
<td>124</td>
<td>-fold over BL-A, hydrophobicity index a factor, transport concluded to be adsorptive, not receptor mediated</td>
<td>-fold over BL-A, hydrophobicity index a factor, transport concluded to be adsorptive, not receptor mediated</td>
<td>-fold over BL-A, hydrophobicity index a factor, transport concluded to be adsorptive, not receptor mediated</td>
</tr>
<tr>
<td>HT29</td>
<td>Human colon carcinoma</td>
<td>Phoroptin intestinal cell line; useful for studies of intestinal development (+/−glucose)</td>
<td>484</td>
<td>Not used for transcytosis studies</td>
<td>Not used for transcytosis studies</td>
<td>Not used for transcytosis studies</td>
</tr>
<tr>
<td>Primary hepatocytes</td>
<td>Rat liver</td>
<td>Consistently polarized cells after 3–4 days; canaliculi formed</td>
<td>135</td>
<td>plgA</td>
<td>Perinuclear accumulation before delivery to apical</td>
<td>-fold over BL-A, hydrophobicity index a factor, transport concluded to be adsorptive, not receptor mediated</td>
</tr>
<tr>
<td>Primary hepatocyte couplets</td>
<td>Rat liver</td>
<td>Short-term (0–24 h) culture of groups of cells with common bile canaliculi; visual assays used due to small fraction (30%) of couplets</td>
<td>51</td>
<td>HDL cholesterol</td>
<td>Scavenger receptor-B1-mediated uptake at B surface and selective A transcytosis of lipid</td>
<td>-fold over BL-A, hydrophobicity index a factor, transport concluded to be adsorptive, not receptor mediated</td>
</tr>
<tr>
<td>Same</td>
<td>Same</td>
<td>No filter needed</td>
<td>same</td>
<td>Horseradish peroxidase</td>
<td>BL-A transcytosis via tubulovesicular intermediates</td>
<td>-fold over BL-A, hydrophobicity index a factor, transport concluded to be adsorptive, not receptor mediated</td>
</tr>
<tr>
<td>HEP-G2</td>
<td>Human hepatocellular carcinoma</td>
<td>Variable fraction of polarized cells</td>
<td>3</td>
<td>NBD-phosphatidylcholine and ergosterol plgA</td>
<td>Vesicular and nonvesicular BL-A transport</td>
<td>Vesicular and nonvesicular BL-A transport</td>
</tr>
<tr>
<td>WIF-B</td>
<td>Rat hepatoma (Pao)-human fibroblast (Wl-38) hybrid</td>
<td>Up to 90% polarized cells, apical cysts form, not canaliculi</td>
<td>83, 521</td>
<td>Endogenous and exogenous apical PM proteins</td>
<td>MAL-2 required for BL-A transcytosis</td>
<td>Use of antibodies to measure BL-A transcytosis</td>
</tr>
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<tr>
<td>Madin-Darby canine kidney (MDCK)</td>
<td>MDCK I, high resistance TER 5–10,000; collecting duct features</td>
<td>209, 470</td>
<td>0.4-μm pore Transwell filters; cells used 3–4 days after seeding; &gt;2,000 TER</td>
<td>Various fluid-phase markers [e.g., Lucifer yellow, HRP, Texas red-dextran (10 kDa)]</td>
<td>Comprehensive biochemical and morphological analysis of endocytosis, recycling and transcytosis from both A and BL</td>
<td>44,605</td>
</tr>
<tr>
<td></td>
<td>MDCK II, low resistance (TER of 150), some proximal tubule features, but no megalin, limited brush border</td>
<td></td>
<td>0.4-μm pore Transwell filters; cells used 3–5 days after seeding</td>
<td>Exogenous rabbit pIgA-R and ligands (anti-pIgA-R IgG, dIgA)</td>
<td>Multiple studies; BLA transcytosis of dIgA rapid (30 min)</td>
<td>390</td>
</tr>
<tr>
<td></td>
<td>3-μm pore collagen-coated PET and 0.4-μm pore polycarbonate filters</td>
<td></td>
<td>Human pIgA-R</td>
<td>IgM and IgA bound, translocated and released equally; therefore, preference for IgA in vivo thought to be ECM barrier to IgM pentamers’ diffusion</td>
<td>397</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Human FcRn</td>
<td>Synthesis and tracking of endogenous FcRn; nonvectorial targeting of newly synthesized with repeated rounds of A-BL and reverse; cytoplasmic dileucine important for transcytosis</td>
<td>454, 556</td>
<td></td>
</tr>
<tr>
<td>Immortalized rat proximal tubule cells (RPT)</td>
<td>tsSV40 transformed; proximal tubule proteins expressed (glut2, megalin); TER of 140; [3H]mannitol permeability of 1.4/40% (+/– cells)</td>
<td>569</td>
<td>3-μm pore high density PET filter</td>
<td>Retinol-binding protein-vitamin A</td>
<td>Megalin-mediated; 20-fold greater A-BL than BLA transcytosis of vitamin A</td>
<td>347</td>
</tr>
<tr>
<td>LLC-PK</td>
<td>Male pig</td>
<td>243</td>
<td>0.4-μm pore Transwell filter</td>
<td>Vitamin B_{12}TCII</td>
<td>TCI degraded ~60% B_{12} stays inside; 20–25% B_{12} complexed to newly synthesized TCI or haptocorrin goes A; 8–12% B_{12} goes BL</td>
<td>405</td>
</tr>
<tr>
<td></td>
<td>Proximal tubule cells; lack μB adapter subunit</td>
<td></td>
<td>50% of apically internalized conjugate moved A-BL; conjugate transported</td>
<td>57Co-B12-IF</td>
<td>100% A-BL of intact cargo via cubilin; LLC-PK and MDCK have much less to no cubilin</td>
<td>462</td>
</tr>
<tr>
<td>OK</td>
<td>Oppossum</td>
<td>293</td>
<td>Millicell-HA filter; cells used 14 days after seeding</td>
<td>50Co-B12-IF</td>
<td>100% A-BL of intact cargo via cubilin; LLC-PK and MDCK have much less to no cubilin</td>
<td></td>
</tr>
<tr>
<td>Primary tracheal cells</td>
<td>Guinea pig</td>
<td>110</td>
<td>Transwell-COL filter; cells used &gt;4 days after seeding; air-liquid interface</td>
<td>Albumin, 70-kDa dextran</td>
<td>Albumin transported A-BL 10-fold greater than BLA; dextran not transported</td>
<td>110</td>
</tr>
<tr>
<td>Primary Clara cells</td>
<td>Rat</td>
<td>76</td>
<td>Tissue culture-treated polycarbonate filter</td>
<td>TH-HRP</td>
<td>~50% of apically internalized conjugate moved A-BL; conjugate transported -&gt; HRP alone</td>
<td>123</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Used after 48 h; not filter grown</td>
<td>Albumin</td>
<td>Saturable endocytosis; overlap with caveolin not lysosome markers; which Clara cell type studying? in vivo clearance also</td>
<td>357</td>
</tr>
<tr>
<td>A549</td>
<td>Human type II-like cells</td>
<td>188</td>
<td></td>
<td>Peptide; proteins and dextrans 1,000–150,000 mol wt</td>
<td>Permeability same at 4 and 37°C, indicating paracellular transport</td>
<td>291</td>
</tr>
<tr>
<td>Calu3</td>
<td>Human bronchial cell phenotype</td>
<td>153, 522</td>
<td>0.4-μm pore Transwell filter, 700–2500 TER</td>
<td>IgA</td>
<td>BL-A upregulated by IFN-γ</td>
<td>330</td>
</tr>
<tr>
<td>Detroit 562 cells</td>
<td>Human pharyngeal carcinoma</td>
<td>441</td>
<td>0.4-μm pore Transwell filter</td>
<td>Strept pneumococci</td>
<td>A-BL via hIgA-receptor; in vivo correlation and MDCK cell also studied</td>
<td>549</td>
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<tr>
<td>Primary placental syncytiotrophoblasts</td>
<td>Normal human term placenta</td>
<td>Cytotrophoblasts isolated and cultured; keratinocyte growth medium induces syncytial formation; alkaline phosphatase expressed on apical surface</td>
<td>39, 283, 288</td>
<td>Millicell filter</td>
<td>IgG</td>
<td>10% of apically applied transcytosed in 60 min; A-BL &gt;&gt; BL-A - 10-fold; IBP transcytosis = in both directions</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.45-µm pore Millicell-HEA filter</td>
<td>LDL and IgG</td>
<td>LDL-Au to lysosomes and IgG-Au to basal tubular elements</td>
</tr>
<tr>
<td>BeWo</td>
<td>Human chorionic carcinoma</td>
<td>Formation of syncytial cells that produce placental hormones and have apical brush border; TER of 60</td>
<td>436</td>
<td>0.4-µm pore low-density filter; cells used 7 days after seeding at subconfluence</td>
<td>FITC-IgG and FITC-dextran (70 kDa)</td>
<td>A-BL of hIgG mediated by FcRn and 3.5-fold greater than BL-A; dextran both directions, much less compared A-BL of IgG in MDCK +/- placental alkaline phosphatase expression to BeWo; A-BL IgG in MDCK low +/- PLAP; A-BL IgG much higher in BeWo; conclusion, not PLAP-mediated</td>
</tr>
<tr>
<td>Primary endothelial cells</td>
<td>Human placenta</td>
<td></td>
<td>266</td>
<td>0.4-µm pore Transwell COL filter; cells used 4 days after seeding; TER of 600</td>
<td>IgG</td>
<td>BL-A via FcRn</td>
</tr>
<tr>
<td>Primary culture</td>
<td>Adult pig</td>
<td>Expression of thyroid-specific genes</td>
<td>363</td>
<td>Grown on floating collagen gels</td>
<td>Thyroglobulin</td>
<td>A-BL movement of intact protein via megalin-mediated endocytosis and avoidance of lysosome</td>
</tr>
<tr>
<td>Same</td>
<td>Human</td>
<td>TER of 1,500</td>
<td>405</td>
<td>Transwell filter + TSH, insulin permeability low</td>
<td>Cationized ferritin None</td>
<td>Injected into colloidal space</td>
</tr>
<tr>
<td>FRT</td>
<td>Fischer rat thyroid</td>
<td>Polarized but no thyroid-specific gene expression; TER of 10,000</td>
<td>406</td>
<td>Transwell filter; cells used 1-7 days after confluency</td>
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<td>Biotinylation</td>
</tr>
<tr>
<td>FRTL-5</td>
<td>Clone of FRT</td>
<td>Question about polarity</td>
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<td>Thyroglobulin</td>
<td>Megalin-mediated transcytosis</td>
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<td>Mammary alveolar epithelial cells</td>
<td>Rabbit midpregnant gland</td>
<td>Lost expression of tissue-specific genes (e.g., whey protein)</td>
<td>174</td>
<td>plgA-R-transfected cells seeded on 0.45-µm pore Transwell filters, coated with types I, III, or IV collagen</td>
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<td>Two cell layers with phenotypes similar to alveolar and myoepithelial cells; TER of 200-10,000; ~6-fold BL-A over A-BL transcytosis</td>
</tr>
<tr>
<td></td>
<td>immortalized with SV40</td>
<td></td>
<td></td>
<td>0.4-µm pore Matrigel-coated polycarbonate filters; plus retinoic acid for 7 days at permissive then 36 h at nonpermissive</td>
<td>Newly synthesized exogenous apical PM protein</td>
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<td>RPE-J</td>
<td>Retinal pigment epithelial cells immortalized with tsSV40</td>
<td>TER of ~300</td>
<td>396</td>
<td>0.4-µm pore Millicell filter</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
molecule can change its biological properties, we assert that one must determine if those changes have altered the molecule’s qualitative or quantitative behavior. Unfortunately, many researchers assume that the modified molecule is normal, which can lead to erroneous or, at the least, uncertain results.

In the remainder of this section we highlight in vitro cell systems currently used for studies of transcytosis. Table 4 presents a partial list. We particularly focus on the extent to which a particular cell line being used and the cargo being followed represent good physiological models. In several cases, there is the need for development and/or improvement of cell models.

B. Microvascular Endothelial Cell Models

Cultured endothelial cells originating from various tissues and vessels abound (155), but few have been used to study transcytosis in vitro. Why? There seem to be two reasons. First, primary endothelial cells show multiple changes after isolation, including a decreased number of caveolar profiles, increased permeability (101), and even transformation from a continuous to fenestrated phenotype (477). These phenomena point to the plasticity of the cells and reinforce the notion that local context dictates the physiology of the vascular bed (538). A second reason is that physical forces (osmotic pressure, flow) in vitro are totally different from those in vivo. Nonetheless, selected aspects of endothelial cell transcytosis have been successfully studied in vitro (Table 4). For example, mechanistic aspects of the albumin receptor, which stimulates fluid-phase transcytosis, have been studied in vitro by Malik and colleagues (13, 89, 379, 576, 602 and discussed below). Interestingly, although the binding/uptake of modified LDLs is commonly used to characterize endothelial cells, and even has been used to purify them (606), to our knowledge there has not been a comprehensive in vitro analysis of the dynamics of the different scavenger receptors (558) or their cargoes. Are the diverse modified LDLs degraded via the lysosomal system and/or transcytosed? Are there regional differences among endothelial cell responses to modified LDLs in vivo? A fruitful area for future research might be to map the surface distributions of the endothelial scavenger receptors, LOX and CD36 (caveolae or clathrin-coated pits?), and determine the intracellular/transcellular fates of the cargoes. Considerable work has been done (2, 36, 108, 296, 326, 399, 461, 496, 606), but we still lack an understanding of the role played by the endothelium in handling atherogenic particles.

Several reasonable in vitro models of brain endothelial cells have been reported (105, 113, 201). Kim (286) has used primary cells and secondary lines from rat and human cerebral cortex to study bacterial adherence and
penetration. One example is in Reference 407. Dehouck and colleagues (85, 114–116) developed a coculture system that appears to represent a good in vitro model of brain capillary endothelium. In brief, freshly isolated astrocytes from newborn rat cerebral cortex are plated on the bottom side of commercial filters (or the bottom of a dish) coated with rat tail collagen, cultured for 3 wk, at which time a subconfluent monolayer of adult bovine brain endothelial cells is plated on the upper side of the filters. After 8 days, confluent endothelial monolayers have formed, and only those exhibiting >500 Ω·cm² are used. In a series of articles, these researchers have characterized the coculture system and validated its use for in vitro studies of brain capillary permeability. The endothelial cells express all of the standard markers of brain endothelial cells and filters. After 8 days, confluent endothelial monolayers have formed, and only those exhibiting >500 Ω·cm² are used. In a series of articles, these researchers have characterized the coculture system and validated its use for in vitro studies of brain capillary permeability. The endothelial cells express all of the standard markers of brain endothelial cells, such as γ-glutamyl transpeptidase, P-glycoprotein, and glucose transporters. Furthermore, a comparison of drug transfer in vivo to that in this in vitro system comes out favorably.

Several studies from this group deserve further comment. Earlier in this review, we referred to the conflicting evidence for and against the quantitative transcytosis of plasma Tf when iron is delivered to the brain interstitium. Results from the in vitro brain endothelial system showed that Tf is transcytosed with iron to the abluminal (basal) side of the monolayer (122). However, the subsequent fates of both components still remain a mystery. The story of the brain endothelial cell LDL receptor is equally puzzling. In confluent endothelial cells (i.e., with underlying astrocytes), LDL is transcytosed in an apical to basolateral direction via LDL receptor residing in caveolae. In contrast, in growing cells, LDL is endocytosed via receptors in clathrin-coated pits, the protein components are degraded, and cholesterol is subsequently used by the endothelial cells themselves (111, 112). The molecular basis for this switch, from receptor-mediated endocytosis of LDL for intracellular utilization to receptor-mediated transcytosis of LDL for use by interstitial cells, is currently unknown. Its elucidation should yield important insights into the machinery required for each type of vesicle traffic.

C. Epithelial Cell Models

1. Intestine

Three cell models of the intestinal epithelium have been used to study the transcytosis of macromolecules ranging from endogenous apical PM proteins and toxins to bacteria (Table 4). Caco-2 cells are the most commonly used because they differentiate furthest along the crypt-to-villus axis and are the easiest to transfect. As mentioned above, the intestinal differentiation program takes up to 20 days in vitro. However, even the most differentiated Caco-2 cells lack the thick glycocalyx expressed by mature enterocytes in vivo; this apical surface feature is considered by some to represent a functional barrier to the adherence and invasion of the intestinal mucosa by many pathogens (160). Therefore, its absence in Caco-2 cells needs to be considered when extrapolating from in vitro results to in vivo possibilities (8). Furthermore, the apparent bidirectional transcytosis of some ligands (e.g., of a transferrin-insulin conjugate, Table 4) may represent reduced polarity in vitro. Interestingly, by coculturing lymphocytes in the basal medium with Caco-2 cells on a filter, the latter have been induced to express M cell-like morphology and activity (284). This achievement has exciting implications for the study of pathogen invasion in vitro (513). Let’s hope that the model is further developed.

HT29 cells can also differentiate along the enterocyte pathway to a mid-mature state, and they additionally have the capacity to differentiate into goblet cells (637). However, HT29 cells are not as easily transfected as Caco-2 cells, nor do they form as tight a monolayer; thus the latter cells are preferred for studies of transcytosis in vitro. The third intestinal cell model, T84, is arrested at an immature enterocyte stage, similar to a crypt cell in vivo. They have been used recently to study IgG transcytosis (Table 4). Additional in vitro models are under development (e.g., Ref. 425a).

2. Liver

The dissociation of liver tissue into isolated cells that were suitable for culture was first described over 30 years ago (37). However, the loss of structural polarity and mixing of membrane domains made them poor models for studies of polarity. The development of isolated couplets was a significant advance for short-term studies of polarized hepatocyte functions, such as transcytosis and bile formation (50). As the importance of the extracellular matrix in maintaining gene expression and promoting cell repolarization became apparent, investigators began culturing hepatocytes on different matrix components in various geometries and physical states. The most successful reconstitution of polarity has come from the use of collagen gels or Matrigel in a sandwich configuration (194, 328). Despite these advances, the limited lifespan of primary hepatocytes and the difficulties in reproducibly obtaining such polarized cultures prompted the use of secondary hepatocyte cell lines.

A) WIF-B Cells. Cassio and colleagues (83, 84) have generated many somatic cell hybrid lines in their studies of the genetic basis of liver-specific gene expression. The WIF-B cells that we currently use were generated by fusion of the differentiated (i.e., expressing liver-specific genes) but nonpolarized rat hepatoma cells (Fao) with human skin fibroblasts (WI-38 cells). Clonal selections ultimately yielded a polarized hepatic phenotype, the WIF12–1 cells, which exhibited a maximum apical polar-
ity of 40% that is, a maximum of 40% of cells in a mature culture formed apical poles (or cysts) with adjacent cells. In collaboration with Cassio, we performed further selections and generated the WIF-B cells, whose apical polarity index is 80% (521).

Before using these cells for membrane traffic studies, we characterized their mature phenotype, especially with regard to cell polarity (251). Most proteins show the same distribution as in vivo, as does microtubule organization, at least near the apical PM (374). Microtubules radiate from the apical membrane, with actin and foci of γ-tubulin also concentrated in this region. The presence of γ-tubulin leads us to conclude that the minus ends of polarized microtubules are closest to the underlying apical PM. We also explored the structural and functional properties of the tight junction boundary (251). ZO-1, the tight junction-associated protein, marks the boundary between the two PM domains. Using short chain lipid analogs in living cells, we established that the tight junctions were an effective “fence” prohibiting the lateral mixing of outer leaflet PM lipids. Surface-labeling of living cells with sNHS-LC-biotin (557 Da) indicated that small molecules had access to the entire PM, while streptavidin (60 kDa) was restricted to only the basolateral domain, establishing that the tight junctions also provided an effective barrier to the diffusion of large molecules into the apical space.

B) HEP-G2 CELLS. The human hepatoma Hep-G2 is currently being used for studies of polarized membrane traffic (Table 4). This line, which expresses many liver-specific genes, was generated in the late 1970s from liver tumor biopsies in which the histology presented as “a well-differentiated hepatocellular carcinoma with a trabecular pattern” (3). The existence of bile canalicular-like cysts within and between cultured HepG2 cells was reported (90); EM observations and one apical PM marker were used in this study. Unfortunately, neither a systematic characterization of these cells nor the culture conditions giving maximum polarity have been performed.

In addition to membrane protein trafficking, lipid trafficking has been actively studied in polarized HepG2 and to limited extent in WIF-B cells and hepatocyte couterparts (102, 198, 213, 233–235, 251, 364, 626, 627). This focus stems from the fact that the major exocrine function of hepatic cells is secretion of bile, whose principal nonprotein components are phosphatidylcholine (PC), cholesterol, and bile salts. To date, lipid analogs have been used in these studies. Given the recent identification of transporters and carriers implicated in cholesterol and phospholipid transport (reviewed in Refs. 46, 138, 150a, 256) and the interest in sphingolipids as possible organizers of lipid microdomains (reviewed in Ref. 237), our understanding of the molecular bases of the vesicular and nonvesicular transcytotic pathways between the basolateral and apical domains of polarized hepatocytes is certain to increase in the next few years. However, it is important to remember that results from studies using in vitro cell models and artificial lipids as surrogates need to be confirmed by in vivo studies.

One obvious feature of polarized hepatocytes is that they sequester their apical surface away from the substrate or bathing medium. This structural organization prohibits direct access to the apical PM, which is confined between adjacent cells and bounded by tight junctions. Hence, quantitative determinations of the polarity index, TER, or rates and extents of PM protein transport to or between the two PM domains using accepted methods (e.g., surface biotinylation as in simple epithelial cells) are not feasible. However, Hoekstra and colleagues (596) have reported methods for differentially measuring fluorescent lipid analogs present at the two PM domains of Hep-G2 cells. We have developed methods in WIF-B cells for detection of trafficked antibodies at the apical PM (250) and proteins secreted into the apical lumen (34).

3. Kidney

As discussed in section II, there is little evidence for in vivo transcytosis of macromolecular cargo in kidney. Nonetheless, MDCK cells, which are derived from dog kidney, are the most-studied epithelial cell model and have been used extensively to study transcytosis (Table 4). These cells were originally developed by nephrologists for permeability and electrical studies. Their subsequent use by cell biologists (86) for studies of the formation of tight junctions, establishment of polarity, and vesicle traffic have popularized MDCK cells to the point that they are now the “NIH-3T3 fibroblast” of the epithelial field. An advantage is that MDCK cells are easily cultured, easily transfected, and become polarized 3–5 days after seeding. They were used in the now classical studies showing that enveloped viruses bud in a polarized fashion and that the newly synthesized viral membrane glycoproteins are targeted directly from the TGN to the appropriate PM domain (480). Furthermore, much of our current understanding of the IgA transcytotic pathway and the sorting signals in the pIgA-R comes from the elegant studies performed in MDCK cells by Mostov and colleagues (393, 394). We expand on this latter topic in section iv.

Two MDCK strains with very different features were identified some time ago (Table 4). The MDCK I cell has a high TER and characteristics reminiscent of the renal collecting duct, whereas the more commonly used MDCK II strain, whose TER is one order of magnitude lower than that of MDCK I cells, has phenotypic features closer to those of the renal proximal tubule. Our own anecdotal experience with MDCK II cells indicates to us that there are many cell variants, some well-documented (367) and others not. Tsukita and colleagues (166) recently reported that stable expression of exogenous claudin 2 in MDCK I
cells lowers its TER to that of MDCK II cells, which express endogenous claudin 2. Does the expression of this tight junction family member in MDCK II cells induce morphological and biochemical changes beyond those contributing to the change in TER?

The first comprehensive and quantitative analysis of apical and basal endocytosis in polarized epithelial cells was performed by Simons and colleagues on MDCK I cells (44, 605). In that study, fluid-phase endocytosis from the apical PM was much less robust than from the basolateral PM. However, apically endocytosed material was transcytosed basally to a relatively greater extent than the converse (material taken up from the basal surface transcytosed to apically). Without knowing the equivalent in vivo endocytic and transcytotic extents at the two poles, this information is hard to interpret.

The apical-to-basolateral “transcytosis” of micronutrients by the proximal tubules of the kidney in vivo makes this topic appropriate for study in kidney cell lines. An important requirement is that the cells express the apical scavenger receptors cubilin and megalin, which have been implicated in the retrieval mechanism (Table 4). Unfortunately, MDCK cells appear not to express these receptors while OK and LLC-PK cells do. The latter cells have been used for studies of vesicle traffic (109, 334). We favor the rat proximal tubule line (IRPT, Table 4), because it expresses functional levels of megalin, and useful reagents are available to rat proteins.

4. Additional epithelial cell systems

A) LUNG. Both primary cells and cell lines, alone and in coculture with endothelial cells, are being used to study transcytosis (Table 4). Calu-3 cells, derived from the upper airways, form tight monolayers and have been found to express and secrete IgA. It would be interesting to know if they also express FcRn and transport IgG in a polarized fashion. Could they be useful for therapeutic drug delivery studies? Likewise, the fact that alveolar type II cells are capable of trans-differentiating into type I cells under defined conditions may make it possible to sort out the confusion over which cell type, if either, is able to transcytose albumin from the thin epithelial fluid layer in the alveolar lumen to the interstitium. Although type I cells contain a huge number of caveolae, Malik and colleagues (267) have recently reported that it is the type II cell that transcytoses albumin via the gp60 membrane protein and caveolae.

B) PLACENTA. Both primary cells originating from protease dissociation of human term placenta and a human placental cell line, BeWo, have been used in in vitro studies of placental transcytosis (Table 4). Under appropriate culture conditions, these preparations differentiate from single cell cytotrophoblasts to multicellular syncytiotrophoblasts. However, the variable TER values reported suggest that different culture conditions are being used that could affect the monolayer integrity of the BeWo cells (Table 4); furthermore, the cell line’s fidelity to syncytiotrophoblasts in vivo is still not totally characterized. Nonetheless, two predominant in vivo cargoes, IgG and iron, are being successfully studied and the molecules involved in their transcytosis identified. B12 and cholesterol are also supplied maternally, yet have not been studied in the isolated placenta. Clearly, there is fertile ground for future work in this system.

C) THYROID. Culture of primary thyroid follicular cells has been reasonably straightforward (Table 4). Additionally, a rat thyroid cell line, FRT, was generated and several sublines exist, with varying degrees of thyroid-specific expression. Both endogenous PM protein and thyroglobulin pathways have been studied with interesting results; that is, Zurzolo et al. (636) reported that immature FRT cells use transcytosis to deliver apical PM proteins, while mature cells use the direct TGN-to-apical route. The mechanistic differences in the two pathways could be identified using such model.

D) MAMMARY. Although it is important to explore the mechanism by which plasma constituents reach the milk, the culture of primary mammary epithelium from lactating glands for such studies has not been reported (382). Because these cells contain and secrete fat globules, they are apparently very difficult to establish in culture and maintain in a differentiated state.

D. Transcytosis Outside of the Epithelial World

1. Bone-resorbing osteoclasts

In the final part of this section, we present a fascinating in vitro transcytotic system that probably operates in vivo but has not yet been confirmed. Bone is a dynamic tissue that it is constantly being laid down and resorbed. Bone degrading cells, osteoclasts, become polarized under appropriate hormonal stimulation and set up an elaborate, sealed structure, called a lacuna, against a segment of bone (572, 585). The cells then secrete hydrolytic enzymes and acid into this extracellular lacuna, which is lined by a ruffled border with membrane markers of lysosomes/late endosomes. Inside the lacuna, the bone mineral content (principally Ca and phosphate) is solubilized and collagen I is partially degraded. These components are then endocytosed at the ruffled membrane front, transcytosed across the cell to a secretory zone, an apical PM domain in the middle of an otherwise basolateral PM (398, 490), and secreted into the marrow cavity. Subsequently, the products are carried into the circulation. Since these cells lack tight junctions, a morphological hallmark in epithelial cells, the system is ideal for studying the establishment of a dynamic polarity in the absence of junc-
tional elements. Furthermore, the transcytotic pathway in these cells differs from that of intestinal epithelial cells, because the cargo is carried from a late endosome (the ruffled border) to the apical PM.

2. Neurons

It is relevant in this section to discuss the possible existence of transcytosis in nerve. Early studies in cultured neurons suggested that the dendritic and axonal PM domains in this polarized cell are functionally equivalent to the respective basolateral and apical PM domains of epithelial cells (544). Carrying this analogy a step further, Dotti and co-workers (221) asked if transcytosis also occurred. Using hippocampal cultures, the researchers found evidence for limited transcytosis of Tf and its receptor from dendrites to axons (221). Given the subsequent work on targeting of native and foreign PM proteins (68, 264, 533), it seems that transcytosis is a minor activity in vitro; its occurrence in vivo is questionable.

IV. MORE ABOUT TWO DIFFERENT TRANSCYTOSIS SYSTEMS

A. Caveolae-Mediated Transcytosis

Transendothelial transport differs in several ways from other types of transcytosis. The transport is rapid (~30 s), the cargo is predominantly fluid not receptor-bound, and a unique vesicle acts as the shuttle between apical and basal surfaces. Despite these differences, the targeting and fusion machinery are reported to be similar to those used elsewhere (see sect. v). In this section, we briefly summarize early work then focus on recent developments that raise new questions about caveolae-mediated transcytosis in endothelium.

1. The endothelial cell surface

The blood-endothelial cell interface is complex in composition and dynamic in its functions (Fig. 5). The apical PM outside of caveolae is negatively charged and thus capable of repelling negatively charged blood cells. Early in vivo ultrastructural studies of endothelium throughout the vascular tree established that sialo- and glycosaminoglycans (principally heparan sulfate proteoglycans) provided the negative charge barrier. Combinations of enzymatic treatments, lectins, and tracers of different pH values were used (537, 540). Clathrin-coated membranes and pits along the apical and basal surfaces of endothelial cells were also negatively charged. Thus cationic molecules were found to preferentially bind at these sites with subsequent delivery to and degradation in lysosomes (536). In contrast, anionic molecules, which include most plasma proteins, were excluded from this pathway. Instead, they were included in the fluid internalized by apical PM caveolae, shuttled to the basal side, and released into the interstitium.

2. Caveolae and caveolin

As stated earlier and shown in Figure 1, caveolae are flask-shaped pits present on and continuous with the apical and basal PMs of all endothelial cells. Although nearly ubiquitous, they are most abundant in terminally differentiated cells, such as adipocytes, smooth muscle cells, type I pneumocytes and, of course, endothelial cells throughout the vascular tree. Even in the early 1980s, endothelial cell caveolae were recognized as chemically distinct “microdomains” of the PM (Fig. 5). Numerous excellent reviews cover caveolar structure and function in greater detail than we can (9, 546, 553a). Our focus is on their role in transcytosis.

As stated in section ii, there is still considerable debate over whether caveolae mediate transcytosis in endothelial cells. Perhaps much of this conflict can be put to rest now that caveolin-1-deficient mice have been generated (130, 465). In animals lacking caveolin-1, the major structural component of caveolae in many tissues including endothelia, no identifiable PM-associated caveolae were observed. In cultured fibroblasts from transgenic embryos, internalization of albumin was inhibited whereas transferrin uptake by clathrin-coated vesicles was not changed (465). Furthermore, and perhaps most significantly, albumin delivery from blood vessel lumens to the underlying interstitium was completely inhibited in perfused transgenic mice (512). Morphologically, gold-labeled BSA was found only in the lumen of caveolin-1-deficient blood vessels, whereas in control endothelial cells, the marker was concentrated at caveolar PM invaginations and in internalized caveolae that were detached and closed from the PM. In aortic ring segments isolated from control or caveolin-1-deficient mice, iodinated albumin transcytosis was measured. Robust transport was seen in control mice, whereas transcytosis was virtually eliminated in the knock-out mice. Interestingly, no accumulations of the tracer during longer incubations were observed in the transgenic mice, suggesting that albumin was not being internalized via clathrin-coated vesicles. However, altered extravascular oncotic pressure normally associated with albumin transcytosis defects was not observed in these animals (130), nor was there any change in cerebrospinal fluid albumin concentrations, a transport pathway thought to be caveolae mediated. These results along with the surprising result that these animals are viable and fertile suggest other compensatory transport processes must be present. Nonetheless, the data strongly indicate that caveolae do indeed mediate transcytosis in endothelial cells.

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Despite these major advances, there are still many unresolved issues. Although caveolae are the likely transcytotic carriers, it is not clear how they transport molecules across the endothelial cell layer. Do caveolae, like clathrin-coated vesicles, pinch off from the PM, travel the short distance across the cell, and fuse with the opposite surface? N-ethylmaleimide (NEM) sensitivity of transport and the identification of caveolae-associated dynamin and specific members of the vesicle docking and fusion machinery (see sect. v) are consistent with this idea. In particular, Schnitzer and colleagues (416) have reported that dynamin activity leads to the scission of caveolae from endothelial PM. Also, EM of albumin-containing free caveolae and caveolae associated with both the basolateral and apical surfaces of the cell are consistent with caveolae as vesicular shuttles (183–185, 378). Alternatively, caveolae on opposing membrane surfaces may transiently (?) fuse with each other producing channels through which ligands pass (Fig. 4). Such structures that contain transcytosing albumin have been occasionally observed at the ultrastructural level (183). One possibility is that both mechanisms operate but mediate the transport of specific ligands or ligands differentially modified. Since more caveolae are observed than the predicted number of pores, the question as to whether all caveolae are transcytosis-competent is also raised. Are some caveolae or
channels reserved for transport while others serve as signaling centers? If so, how are they different?

3. Albumin and orosomucoid transcytosis

Although most transcytosed cargo in endothelial cells is fluid, two of the best-studied molecules, albumin and orosomucoid, are transported predominantly via receptor-mediated mechanisms. The first clues that albumin was internalized via receptors came from morphological studies where albumin association with endothelial cells was concentrated in caveolae open to blood vessel lumens (183, 184, 378). Three major albumin binding proteins were subsequently identified (p18, p31, and p58–60) that were present on endothelial luminal cell surfaces (179, 180, 507, 508). Further work revealed that p18 and p31 were likely scavenger receptors and that gp60 was the bona fide albumin receptor (506, 575). Albumin binding to gp60 and gp60 antibody cross-linking activates the trans-endothelial transport of albumin in vitro and in vivo (602).

In both cases, the tyrosine phosphorylation of gp60 itself, caveolin-1, and the tyrosine kinases pp60 src and Fyn were induced upon activation. Addition of the specific tyrosine kinase inhibitors herbimycin and genistein blocked albumin internalization, indicating that transcytosis is a tyrosine kinase-dependent process (576). More recently, this activation has been further correlated with a Gαs-coupled src kinase signaling pathway that is discussed below (see sect. vD1).

Like albumin, when examined ultrastructurally, transport of orosomucoid is rapid and occurs via caveolae (456). Previous studies showed that orosomucoid binding to cultured endothelial cells was saturable, specific, and high affinity, implying the presence of specific receptors (504). Ligand blotting of endothelial cell lysates with 125I-orosomucoid identified three receptor candidates of 14, 20, and 7 kDa that have not been examined further (456). Like albumin, orosomucoid transcytosis is also NEM sensitive, consistent with the identification of caveolae as vesicle shuttles between the two domains during transcytosis (456). Because none of these possible receptors or gp60 has been cloned, the structural signals that direct transcytosis of these molecules and their associated ligands have not yet been identified. Will there be any similarities to those identified in plgA-R (see below)? What dictates their placement in caveolae over clathrin-coated pits?

B. Clathrin-Mediated Transcytosis

As discussed in section vC3, one of the best-studied examples of transcytosis emerged from efforts aimed at understanding the molecular basis of mucosal immunity. Here we will discuss in detail the transcellular itinerary of the plgA-R and its ligand, plgA. Also, we will describe what is understood about the signals encoded in the cytoplasmic tail of the receptor that direct the complex’s circuitous journey. Finally, we will compare the plgA-R transcytotic pathway with that of newly synthesized hepatic and intestinal apical PM residents.

1. The plgA-R transcytotic pathway

The intracellular itinerary that plgA-R follows during its life cycle is complex and may vary among epithelial cell types examined and among species (388, 439). Nonetheless, from studies performed in hepatocytes, enterocytes, and their respective in vitro model systems as well as from MDCK cells that stably express exogenous plgA-R, a general picture is emerging that is summarized in Figure 6. From work performed in rat hepatocytes (565), it was determined that plgA-R is synthesized in the ER as a 105-kDa polypeptide and travels to the Golgi where it is terminally glycosylated giving rise to an 116-kDa glycoprotein. This form of the receptor is delivered from the TGN to the basolateral surface where it is further modified (probably phosphorylated, see below) reaching its mature form of 120 kDa. Early in vivo experiments, also performed in hepatocytes, further determined that the basolaterally located receptor is subsequently internalized (in the presence or absence of bound plgA) by clathrin-coated pits and delivered to early endosomes (176, 238). Other receptor-mediated ligand systems (e.g., ASGP-R) were also present in these early endosomes, but only the plgA-R and its ligand were delivered to a subapical compartment before their release into the bile. The other internalized receptor proteins moved along one of two other arms of the endocytic pathway, to lysosomes or back to the basolateral PM.

The transcytotic pathway taken by plgA-R in MDCK cells has been studied extensively with the goal of identifying and characterizing all of the intracellular intermediates. Debate over nomenclature and different experimental approaches led to conflicting views that now appear to be resolved. Recent reports (316, 609) have established that at least three compartments comprise the basolateral-to-apical transcytotic pathway of plgA-R in MDCK cells: basolateral early endosomes, a “common” endosome, and an apical recycling endosome (Fig. 6). In hepatocytes and enterocytes, there are only two identified transcytotic intermediates: basolateral early endosomes found in both cell types and a “common” endosome described in enterocytes or the subapical compartment (SAC) in hepatocytes (29, 250, 290) (Fig. 6). The basolateral early endosomes in the different cell types appear to be rather analogous structures. They are the first entry points for molecules internalized from the basolateral surface, they are biochemically distinct from apical early endosomes (that
are first to receive apically internalized molecules), and morphologically, they are located at the basolateral pole of the cell. The MDCK apical recycling endosome is most similar to the hepatic SAC. Both have a neutral pH, only apically destined membrane components traverse them, and they are located closest to the apical PM. The major difference between them is the absence of recycling apical PM proteins in hepatic SAC and their presence in the MDCK apical recycling endosome. Why such a similar structure is not present (or not yet identified) in enterocytes is a puzzle. Also, why do hepatocytes lack a common endosome (that receives cargo from both cell surfaces)? These differences may be pointing to unique and important differences in membrane transport among epithelial cell types.

Once the receptor has reached the apical cell surface, it is cleaved and its ectodomain is released into the luminal spaces. The receptor is constitutively synthesized and transcytosed whether ligand is present or not such that both the unoccupied SC and secretory IgA are recovered from apical secretions. In hepatocytes, the plgA-R is cleaved efficiently at the apical surface such that the pathway is "one-way"; all receptor molecules that reach the apical cell are clipped. However, in MDCK cells, plgA-R proteolysis is less efficient such that a small proportion of intact plgA-R at the apical cell surface can be recycled, and even a smaller portion can be transcytosed back to the basolateral surface (Fig. 6). Whether this is a physiologically meaningful process is not clear. These MDCK cells are overexpressing the rabbit form of plgA-R, and it is possible that in the canine context, this receptor has altered dynamics. Examination of the dynamics of the very low levels of the endogenous MDCK plgA-R may help clarify this point. Work performed in vitro in nasopharyngeal cells has shown that uncleaved apical plgA-R mediates endocytic entry of Streptococcus pneumoniae, suggesting that apical internalization of plgA-R may be part of its life cycle in certain cell types (273). Identifying the cell surface protease and understanding its proteolytic activity, specificity, and tissue distribution will help us clarify the posttranscytotic fate of the receptor in these other cell types.
2. Signals and regulation of pIgA-R transcytosis

The 103-amino acid cytoplasmic domain of pIgA-R contains multiple signals that mediate its circuitous journey. From work performed mainly in MDCK cells, the signals were found to fall into three categories: targeting to the basolateral PM, promoting rapid endocytosis, or preventing degradation (Fig. 7). With the use of deletion analysis, the pIgA-R basolateral targeting signal was identified in a sequence located just adjacent to the TMD, and this amino acid stretch was sufficient to direct chimeric reporter proteins to the basolateral PM (81). Site-directed mutagenesis of the receptor tail revealed that internalization from the basolateral surface required two cytoplasmic tyrosine residues, both of which are found in sequences similar to previously identified internalization motifs (421). Phosphorylation of serine-726 has also been shown to mediate rapid pIgA-R basolateral internalization, and it has been suggested this modification is reflected in the increased molecular mass (120 kDa) of the basolaterally located receptor (231, 422). Once internalized, the receptor is rapidly transcytosed across epithelial cells, and phosphorylation of serine-664 may facilitate this process. Also, a region of the tail has been shown to prevent the receptor from being degraded further, ensuring its efficient transcytosis once internalized (55). From these studies, it has been proposed that newly synthesized pIgA-R leaves the TGN in a nonphosphorylated form. Upon delivery to the basolateral surface, the receptor is phosphorylated (serine-726), which exposes the tyrosine-based internalization motifs allowing rapid internalization. Subsequent phosphorylation at serine-664 promotes transcytosis, thus sorting the receptor away from the degradative or recycling pathways (388).

Transcytosis of pIgA-R is also regulated upon ligand

---

**TMD**  
**pIgA-R Cytoplasmic Domain (103 aa)**

<table>
<thead>
<tr>
<th>aa</th>
<th>630</th>
<th>653</th>
<th>669</th>
<th>707</th>
<th>755</th>
</tr>
</thead>
</table>

**Basolateral Targeting Signal**

| aa | 653 | 654 | 669 | 670 | 708 |

**Tyrosine-based motif**

| Y668-C | Y734-S |

**Unknown motif**

| (unphosphorylated residue) S726-A |

**Basolateral Internalization Signals**

| 725 | S 737 |

**Transcytosis Signal**

| S664-A (unphosphorylated residue) |

| S664-D (mimics phosphorylated residue) |

---

**Fig. 7.** The targeting signals encoded by the cytoplasmic carboxy-terminal tail of pIgA-R. A schematic drawing is shown summarizing the mutations made in pIgA-R’s cytoplasmic domain and their effects on receptor trafficking. From these studies, the indicated trafficking signals were identified. The more detailed versions of the basolateral targeting and internalization signals were identified in Refs. 19, 20, 421, 422. aa, Amino acid; BL, basolateral; TMD, transmembrane domain.
binding, which significantly enhances its transport from the apical recycling endosome to the apical PM in MDCK cells (548). Ligand binding rapidly causes the tyrosine phosphorylation of many proteins in MDCK cells including phospholipase C-γ1, from which a signal transduction cascade has been proposed (388). Similarly, plgA-R transcytosis in intact hepatocytes is stimulated by intravenous injection of plgA into rats (189). Although the accelerated transport step was not identified, clues to the activated tyrosine kinase came from studies performed in mouse hepatocytes (341). Coimmunoprecipitations showed that p62yes interacts with the plgA-R tail in rat hepatocytes, and in p62yes knockout mice, both basolateral to apical constitutive and activated transcytosis of plgA were impaired (341). However, this mechanism may not be universal, since dlgA does not activate transcytosis in FRT cells that express exogenous plgA-R (493). Also, the human forms of plgA-R do not respond to ligand binding in polarized MDCK cells (all other studies were performed using the rabbit form), further suggesting that mechanisms regulating transcytosis differ among organisms. Careful comparison of cytoplasmic tail sequences may help pinpoint the reasons for the differential regulation.

3. Transcytosis of plgA-R versus newly synthesized apical PM residents

As described in section 1F, hepatocytes and enterocytes use the transcytotic pathway to deliver newly synthesized resident proteins to the apical surface. This “indirect” pathway includes transport of the newly synthesized apical proteins from the TGN to the basolateral PM where they are then selectively endocytosed and transcytosed to the apical PM (33, 499). Are the transcytotic pathways of the plgA-R and apical residents shared? We tested this hypothesis by immunolocalizing newly synthesized apical PM proteins and plgA-R in hepatocytes from bile-duct ligated rats, a condition reported to perturb hepatic IgA transport (29, 306). We found that both the residents and plgA-R accumulated in the SAC. We next applied pulse-chase metabolic labeling combined with subcellular fractionation, vesicle immunolocalization, and immunoprecipitation of apical PM proteins to obtain more quantitative information about this intracellular compartment (30). We found newly synthesized dipeptidyl peptidase IV (DPPIV), a single TMD apical PM protein, first in immunolabeled early endosomes and subsequently in isolated SAC. The high specific radioactivity of DPPIV (i.e., 35S-DPPIV/immunoreactive DPPIV) in this latter vesicle fraction indicated that very little preexisting (unlabeled) DPPIV was present. This meant that recycling of resident apical PM proteins was either very limited or did not involve SAC.

The transcytosing molecules, although ultimately directed to the apical cell surface, must first traverse the basolateral cell surface. Besides the basolateral targeting signal identified for plgA-R, at least two other such signals have been identified. They either contain a tyrosine in the context of a short degenerate sequence or a dileucine motif (21), and in some cases, they overlap with those used at the basolateral PM in receptor-mediated endocytosis. In general, the tyrosine-based motifs are thought to be recognized by the μ-subunit of the Golgi tetrameric adaptor protein-1 (AP-1), whereas the leucine-based signals are thought to interact with the β-subunit (361, 392). Identification of a new epithelial-specific μ1-subunit, μ1B, (157) has added a new twist to basolateral sorting. Its role in TGN to basolateral delivery was proposed when it was observed that introduction of μ1B into LLC-PK1 kidney cells, which lack endogenous μ1B, redirected its mis-sorted LDL receptor (LDL-R) and Tf-R with high fidelity to the basolateral PM (157). Since these cells express the “original” μ1a-isofor, μ1a, the μ1B targeting appeared to be dominant (157). However, the specific transport step at which the μ1B-subunit functions was not identified in this study. More recently, Gan et al. (173) reported that in the cells lacking the μ1B-subunit, TGN to basolateral targeting of LDL-R and Tf-R occurred, but basolateral recycling was impaired, thus μ1B sorts basolateral proteins postendocytically. This is consistent with the observation that basolateral targeting signals function both to deliver molecules to the basolateral domain, but also regulate basolateral internalization and recycling (for example, see Ref. 20). Despite where μ1B functions, it is still a paradox that in liver that lacks μ1B expression, the same basolateral receptors as in LLC-PK1 cells are delivered directly and with high fidelity to the basolateral PM. How do hepatocytes, without functional μ1B/AP-1 complexes, sort proteins to the basolateral surface and maintain them there? What interprets the nontyrosine, nonleucine-based plgA-R basolateral targeting signal? Also, how are the apical residents targeted to the basolateral surface in hepatocytes? Unlike plgA-R, the cytoplasmic tails on several apical ectoenzymes that we have studied are very short (6–8 amino acids), with no identified or nonexistent (the GPI-anchored proteins) signals suggesting that the mechanisms regulating transport must be quite different.

A related puzzle is the mechanism(s) regulating internalization of the apical residents from the basolateral domain. Both newly synthesized plgA-R and apical residents rapidly traverse the endoplasmic reticulum and Golgi and reach the basolateral PM within 45 min (32, 499). However, the rates of their redistribution to the apical PM vary greatly, which might reflect differences in their modes of basolateral internalization. The different cytoplasmic tails may also reflect different internalization routes, and in the case of the apical residents, these may likely be non-clathrin-mediated mechanisms. Alternatively, the proteins are internalized at the same rates and to the same extents. The variable trafficking kinetics in-

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stead may reflect differences in sorting or transport mechanisms elsewhere in the transcytotic pathway (e.g., basolateral early endosomes).

V. MECHANISMS AND MOLECULES REGULATING TRANSCYTOSIS

In this section, we discuss what is known about the mechanisms and molecules that regulate transcytosis in polarized epithelial cells. Are there unique mechanisms/molecules required? Do different cell types require specific molecules or combinations of molecules to perform their specialized transcytotic functions? How does the cell discriminate between transcytotic delivery and self use? Surprisingly, there is little direct evidence for the involvement of specific molecules in transcytosis, but many promising candidates are emerging. We will describe some of these candidates, emphasizing those involved in vesicle transport, targeting, and consumption along the transcytotic route. However, a cautionary flag must be raised. Much of what is discussed is based on studies performed only in tissue culture models of polarized cells (especially MDCK cells) and has not yet been tested in a physiological context. Furthermore, many candidates have been named solely upon their subcellular location; the functional studies have not been performed. Nonetheless, there are exciting possibilities and the groundwork is clearly set to direct future investigations.

A. Targeting Machinery

1. The SNARE hypothesis

How do cargo-bearing vesicles deliver their contents to the correct target domain? Morphological, biochemical, and genetic approaches have successfully identified many molecules involved in “vesicle targeting,” a complex series of molecular events that minimally includes docking and fusion. These approaches indicate that the basic mechanisms are conserved among the membrane compartments throughout the biosynthetic and endocytic pathways and in organisms ranging from yeast to humans. The players so far identified fall into two broad categories: some are used repeatedly throughout the pathways, others belong to discrete protein families, where one or a few members act at one or a few transport sites. Members of three protein families are central players in vesicle targeting and fusion (216, 262, 335, 443). They are as follows: 1) the SNAREs which, in general, are a group of cytoplasmically oriented integral membrane proteins that are present on vesicles (v-SNAREs) or target membranes (t-SNAREs) (also referred to as Q- and R-SNAREs; see Refs. 613 and 144); 2) the Sec1/Munc18 proteins; and 3) small-molecular-weight GTP-binding proteins, the rabs.

The “SNARE hypothesis” emerged to describe the mechanism by which the SNAREs promote membrane docking and fusion through interactions with an ATPase, N-ethylmaleimide sensitive factor (NSF), and its “receptor,” α-SNAP (547), but has undergone considerable revision as more is learned about these and other essential proteins and the cyclical nature of the process is better appreciated (262). For vesicle trafficking to continue past one round, some of the machinery must continually cycle between vesicle and target membranes, making the order of events difficult to define. Also, the t- and v-SNAREs, whose pairwise coupling was originally thought to confer targeting specificity, show promiscuity in their binding in vitro, suggesting that other factors are required for the high fidelity of membrane targeting observed in vivo. Furthermore, the ATPase, NSF, which was originally postulated to function in the fusion reaction itself, most likely functions as a chaperone to disassemble the SNARE pairs (204, 216). Whether this occurs when the pairs are in “trans” (on the cognate membranes) or in “cis” (on the same membrane after vesicle consumption) may differ depending on the cell type or transport step examined.

It is also important to note that the “SNARE hypothesis” developed mainly from studies examining vesicle docking and fusion at the PM where SNAP-25 family members (t-SNARE isoforms) are required. Because these molecules are predominantly PM associated, the mechanisms regulating intracellular vesicle transport are likely different. Consistent with this is the finding that two t-SNAREs (both syntaxins) and two v-SNAREs are required during late endosome fusion, whereas at the PM, two different t-SNAREs (a syntaxin and SNAP-25 protein) and one v-SNARE participate (15). Interestingly, in both cases the molecules form core complexes that are structurally similar, indicating that the mechanisms, albeit different, are conserved. Newer models have integrated the enormous and confusing body of information and have identified common steps in membrane targeting which minimally include 1) SNARE activation (which likely includes NSF chaperone activity) and rab recruitment to proper organelle sites; 2) cognate membrane attachment; and 3) membrane fusion and bilayer mixing (262). The order of events and the mechanistic details are not clear, yet there is considerable evidence that the SNAREs, rabs, and Sec1/Munc18 proteins are key players in the process. In the following sections, we discuss the possible roles of selected members of the transport machinery in regulating transcytosis in polarized epithelial cells. Table 5 summarizes what we currently know.

2. NSF and α-SNAP

NSF was initially implicated as a regulator of polarized PM targeting from studies that examined the effects of NEM on protein transport. Addition of NEM reduced
IgA transcytosis by ~70% and its basolateral targeting by ~90% in permeabilized MDCK cells (17, 332). It also inhibited fusion (as assayed by plgA-R processing) of transcytotic carrier vesicles with the apical PM in a hepatic cell-free system reconstituting the final step of transcytosis (564). Because NSF ATPase activity is inhibited by alkylating (617), it was the likely target for NEM. In support of that, addition of recombinant NSF restored much of the activity lost in MDCK cells or hepatic cell-free systems treated with NEM or anti-NSF antibodies (17, 253, 332, 564). Interestingly, direct apical PM targeting was insensitive to NEM (253, 332), indicating that it requires an unidentified NSF homolog or does not require NSF-like activity at all. Other key factors, the SNAP family of NSF receptors (α-, β-, and γ-isoforms), recruit NSF to organelles and activate its ATPase activity (617). α-SNAP has been identified in polarized epithelial cells and its role in TGN to PM targeting examined in MDCK cells. Addition of α-SNAP antibodies and treatment of cells with botulinum E (an α-SNAP-specific toxin) inhibited direct transport to both domains (17, 253, 300). Unfortunately, its role in transcytosis was not examined.

NSF has also been implicated as an important regulator of transcytosis in endothelial cells. Treatment of myocardial or rat lung endothelium with NEM significantly inhibited transcytosis in situ (80 and 50%, respectively) (455, 505). NSF and its receptor α-SNAP were also found associated with caveolae, supporting the role of this vesicle type as the transcytotic carrier in endothelial cells (509). Furthermore, immunoprecipitation of exogenously added recombinant myc-tagged NSF from rat lung endothelial cell extracts revealed the presence of large, ATP-dependent, NEM-sensitive complexes that contained many members of the transport machinery including α- and γ-SNAP, cellubrevin, syntaxin, and rab5 as well as caveolin and dynamin (457). The complexes were also found to contain cholesterol, the ganglioside GM1, and other unidentified lipids. As predicted, these complexes were immunoprecipitated from membrane extracts, but they were also surprisingly recovered from cytosol preparations. How these supramolecular protein-lipid complexes function in membrane transport is not yet known but supports a role for both caveolae (the complexes contained caveolin) and the molecules of the SNARE hypothesis in endothelial transcytosis. Do the complexes present in the cytosol or on membranes serve the same function? Are similar complexes present in other polarized epithelial cell types?

3. t-SNARES and v-SNARES

There are two families of t-SNARE proteins: the syntaxins and the SNAP-25 family. To date, at least 18 syntaxin family members have been identified in mammalian cells of which 5 (syntaxins 1A, 1B, 2–4) are PM-specific isoforms (262). The SNAP-25 family is much smaller, with only three identified members: SNAP-25, SNAP-23, and SNAP-29 (262). Like the syntaxins, these proteins are relatively small (23–29 kDa), cytoplasmically oriented molecules. Although not integral membrane proteins as are the PM syntaxins, SNAP-25 proteins associate with the bilayer through cysteine-linked palmitoyl chains located near the middle of the protein. Biochemically, SNAP-25 proteins bind PM-associated syntaxins and v-SNAREs in vitro to form the four-stranded α-helical ternary complexes required for vesicle docking and fusion (262).

The distributions of syntaxins 2, 3, and 4 at the PM have been examined in different epithelial cells. In particular, we found that rat hepatocytes express three endogenous PM-associated syntaxin isoforms (syntaxins 2, 3, and 4) and SNAP-23 (163). Quantitative immunoblotting revealed that all four t-SNAREs are relatively abundant in liver (~11–668 nM corresponding to 0.5–28 × 10^5 molecules/cell). Biochemically, each of the t-SNAREs was observed predominantly in hepatocyte PM sheets with overlapping but distinct expression patterns among the PM domains. Both syntaxin 4 and SNAP-23 are restricted to the basolateral PM while syntaxins 2 and 3 are more apically distributed, with greater enrichment of syntaxin 3 in this domain. Despite the biochemical abundance of the molecules, we were able to detect only syntaxins 2 and 4 in rat liver sections in situ. However, the distributions did not fit with our biochemical data; we found syntaxin 4 in both domains. Similar discrepancies were observed in WIF-B cells. Like in liver hepatocytes, syntaxin 3 was at the apical domain, but unlike liver, syntaxin 2 was restricted to the apical domain. Also, syntaxin 4 and SNAP-23 were found in both domains. These varied distributions likely reflect important differences in regulation of PM dynamics between the in vivo and in vitro systems and may point to interesting features of the cellular itineraries and functions of the t-SNAREs. Little is known how these putative targeting molecules are themselves targeted to the correct PM domain, and once delivered, how and if they are retained there.

Interestingly, the PM syntaxins display remarkable variability in their domain distributions in other polarized cells (see Table 5). Only syntaxin 3 appears to be consistent with apical distributions observed in all polarized cells examined (117, 163, 170, 331, 438). SNAP-23 distributes to both PM domains in all but two epithelial cell types (see Table 5). Since it is thought to be required for vesicle docking and fusion, SNAP-23’s uniform PM distribution fits with the current model of its ubiquitous involvement in t- and v-SNARE ternary complex formation. However, its absence at the apical PM in hepatocytes and pancreatic cells is somewhat paradoxical. Either these cells have unique mechanisms for regulating transport at
### TABLE 5. Putative regulators of transcytosis in polarized epithelial cells

<table>
<thead>
<tr>
<th>Protein</th>
<th>Subcellular Location in Epithelial Cells [Morphology (M)/Fractionation (F)]</th>
<th>Epithelial Cells Examined</th>
<th>Evidence for Role in Transcytosis</th>
<th>Reference No.</th>
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<tr>
<td>Syntaxin 2, 3, 4</td>
<td>Apical and/or basolateral PM (M, F)</td>
<td>Hepatocytes, pancreatic acinar and intestinal epithelial cells, kidney collecting duct, Caco-2, MDCK and WIF-B cells</td>
<td>Location; syntaxin 3 overexpression in MDCK and Caco-2 cells and antibody sensitivity in MDCK cells</td>
<td>59, 117, 163, 168, 331, 345, 438</td>
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<tr>
<td>Syntaxin 8, 13</td>
<td>TGN</td>
<td>Hepatocytes, WIF-B cells</td>
<td>Location</td>
<td>562, 583</td>
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<tr>
<td>Syntaxin 6, 10</td>
<td>Apical and/or basolateral PM (M, F), endosomes (M, F)</td>
<td>Hepatocytes, MDCK, WIF-B, and IMCD cells</td>
<td>Location; toxin sensitivity in MDCK and IMCD cells and anti-SNAP23 sensitivity in MDCK cells</td>
<td>26, 88, 169, 255, 314, 333</td>
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<tr>
<td>SNAP 23</td>
<td>Apical and/or basolateral PM (M, F), endosomes (M, F)</td>
<td>Hepatocytes, MDCK, WIF-B, and IMCD cells</td>
<td>Location; toxin sensitivity in MDCK and IMCD cells and anti-SNAP23 sensitivity in MDCK cells</td>
<td>26, 88, 169, 255, 314, 333</td>
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<td>VAMP 2</td>
<td>Endosomes (F)</td>
<td>Gastric parietal, MDCK and IMCD cells</td>
<td>Location</td>
<td>27, 74, 233, 371</td>
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<td>Basolateral endosomes (M, F)</td>
<td>Hepatocytes</td>
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<td>VAMP 7/TG-VAMP</td>
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<td>Caco-2 cells</td>
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<td>VAMP 8/endohorbivin</td>
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<td>NSF</td>
<td>Golgi (M, F), endosomes (F), coated vesicles (F)</td>
<td>Placenta, pinealocytes, MDCK cells</td>
<td>NEM and anti-NSF sensitivity and mutational analysis in MDCK</td>
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<td>Munc 18-2</td>
<td>Apical PM (M)</td>
<td>Murine intestinal, lung, kidney, testis and spleen epithelia, MDCK cells</td>
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<td>a-SNAP</td>
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<td>MDCK cells</td>
<td>Anti-a-SNAP sensitivity in MDCK</td>
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<td>Rab1 and 2</td>
<td>Golgi (F), transcytotic vesicles (F)</td>
<td>Hepatocytes</td>
<td>Copurification with transcytotic vesicles</td>
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<td>Rab3B</td>
<td>Tight junctions (M)</td>
<td>Intestinal and kidney epithelia, epithocytes, HT-29, and T84 cells</td>
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<td>Rab13</td>
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<td>Rab17</td>
<td>Basolateral PM (M), apical vesicles and tubules (M), transcytotic vesicles (F), apical recycling endosome (M)</td>
<td>Hepatocytes, enterocytes, kidney epithelia, MDCK, and Eph4 cells</td>
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<td>Rab25</td>
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<td>MDCK cells</td>
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<td>Annexin II</td>
<td>Apical and/or basolateral PM (M), early endosomes (F), subapical structures (M, F)</td>
<td>Intestinal and mammary epithelia, hepatocytes, MDCK cells</td>
<td>Location; change in distribution upon activation of transcytosis with bile acids in hepatocytes</td>
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<td>Annexin IV</td>
<td>Apical and/or basolateral PM (M, F), subapical structures (M)</td>
<td>Kidney, intestinal, uterine, tracheal and fallopian tube epithelia, T84 cells</td>
<td>Location</td>
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<td>Annexin VI</td>
<td>Apical and/or basolateral PM (M, F), subapical structures (M, F)</td>
<td>Hepatocytes</td>
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<td>Annexin VII</td>
<td>Apical PM (M)</td>
<td>Intestinal epithelia</td>
<td>Intestinal-specific expression; location</td>
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<td>Annexin XIIIa</td>
<td>Apical PM &gt; basolateral PM (M)</td>
<td>Enterocytes and MDCK cells</td>
<td>Location; intestinal-specific expression</td>
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<td>Annexin XIIIb</td>
<td>Apical PM, subapical structures (M, F)</td>
<td>MDCK cells</td>
<td>Location; kidney-specific expression</td>
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<td>Dynamin-1</td>
<td>Caveolae, apical PM (M, F)</td>
<td>Endothelial and MDCK cells</td>
<td>Location; GTP dependence, overexpression and mutational analysis in MDCK cells, anti-dynamin sensitivity in endothelial cells</td>
<td>7, 416</td>
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<tr>
<td>Dynamin-2</td>
<td>Apical and BL PM (M, F)</td>
<td>MDCK cells</td>
<td>Location; GTP dependence, overexpression and mutational analysis in MDCK cells</td>
<td>7</td>
</tr>
<tr>
<td>Cytoplasmic dynein</td>
<td>Golgi (F), endosomes (F)</td>
<td>Intestinal epithelia, hepatocytes</td>
<td>MT dependence and orientation; NEM sensitivity</td>
<td>146, 415</td>
</tr>
<tr>
<td>Myosin I</td>
<td>Zymogen granules (M, F), Golgi (F), apical PM (M, F)</td>
<td>Pancreatic acinar cells, intestinal epithelia, hepatocytes, Caco-2 cells</td>
<td>Location; actin dependence, mutational analysis in Caco-2 cells</td>
<td>25, 94, 136, 145, 440, 452</td>
</tr>
<tr>
<td>Myosin V</td>
<td>Apical PM (M, F), apical recycling endosome (M)</td>
<td>Intestinal epithelia, MDCK and WIF-B cells</td>
<td>Location; actin dependence, mutational analysis in MDCK cells</td>
<td>218, 304, 321</td>
</tr>
<tr>
<td>Myosin VI</td>
<td>Apical PM (M, F)</td>
<td>Intestinal epithelia</td>
<td>Location; actin dependence</td>
<td>215, 218</td>
</tr>
<tr>
<td>RhoA</td>
<td>Basolateral endosomes (M)</td>
<td>MDCK cells</td>
<td>Location; mutational analysis</td>
<td>315</td>
</tr>
<tr>
<td>Rac1</td>
<td>Apical recycling endosomes (?)</td>
<td>MDCK cells</td>
<td>Location; mutational analysis</td>
<td>279</td>
</tr>
</tbody>
</table>

IMCD, inner medullary collecting duct; MT, microtubule; N/D, not determined; TGN, trans-Golgi network; PM, plasma membrane.
their apical surfaces, or other SNAP-25 isoforms are yet to be identified.

To date, the roles of the t-SNAREs in polarized PM targeting have been tested directly mainly in MDCK cells that were stably expressing plgAR and overexpressing wild-type syntaxins 2, 3, or 4 (332). Neither transcytosis nor basolateral transport of plgAR was affected in these cells. Similarly, in Caco-2 cells overexpressing syntaxin 3, no changes in basolateral protein targeting were observed (59). Together, these results suggest that the PM-associated syntaxins do not regulate these transport pathways or that other yet-to-be isoforms are involved. Alternatively, overexpression was not high enough to be inhibitory or does not negatively regulate these processes. However, overexpression of syntaxin 3 in MDCK and Caco-2 cells did lead to alterations in apical PM dynamics. In MDCK cells, a slight impairment (~20%) of direct TGN to apical PM delivery of a chimeric plgAR molecule and IgA was observed as well as apical recycling (also ~20%). Likewise in Caco-2 syntaxin 3 overexpressors, the direct apical targeting of sucrase-isomaltase and the apical secretion of α-glucosidase was significantly impaired. Furthermore, anti-syntaxin 3 antibodies inhibited direct targeting of hemagglutinin (HA) in MDCK cells, confirming a role for this syntaxin in apical delivery (300). Unfortunately, the effects on transcytosis of anti-syntaxin 3 or syntaxin 3 overexpression in Caco-2 cells were not examined. However, the role of SNAP-23 in transcytosis has been examined in MDCK cells by treating with SNAP-23/glucosidase was significantly impaired. Furthermore, anti-syntaxin 3 antibodies inhibited direct targeting of hemagglutinin (HA) in MDCK cells, confirming a role for this syntaxin in apical delivery (300). Unfortunately, the effects on transcytosis of anti-syntaxin 3 or syntaxin 3 overexpression in Caco-2 cells were not examined. However, the role of SNAP-23 in transcytosis has been examined in MDCK cells by treating with SNAP-23/25-specific neurotoxins. In these cells, basolateral to apical transport of plgA was inhibited by 30% (17), as was basolateral targeting of the receptor, but the TGN to apical targeting of HA was not affected (17, 253). More recent studies confirmed this result and found that toxin activity also impairs transferrin recycling (314).

The direct involvement of v-SNAREs in transcytosis has not been explored, but the obvious prediction is that they are required. VAMP 1, 2 and VAMP 3/cellubrevin are ubiquitously expressed, and the presence of VAMP 3/cellubrevin on endosomal structures in hepatocytes and VAMP 2 on endothelial caveolae has been reported (75, 371). VAMP 8/endobrevin is enriched in epithelial tissues and has been localized to the apical pole in kidney epithelium (622). Interestingly, in hepatocytes, this VAMP species was found to be enriched in basolateral early endosomal fractions, whereas in MDCK cells, it was localized to both apical endosomes and the apical PM (554). Whether this protein functions in basolateral or apical PM targeting (or both) is not yet known. In Caco-2 cells, another VAMP isoform, VAMP 7/TI-VAMP, is also localized to both the apical PM and in subapically located structures where it has been proposed to function in the later steps of apical PM delivery (171). Interestingly, this VAMP species has a long NH2-terminal extension that resembles a region in annexin XIIIb, another protein implicated in regulating apical vesicle delivery (150). This sequence in annexin XIIIb encodes a lipid-binding domain, but whether VAMP 7/TI-VAMP shares this biochemical property is not yet known.

A possible role for VAMP 2 in transcytosis has been suggested from studies in SLO-permeabilized rat lung endothelial cells using VAMP-specific neurotoxins (371). In cells treated with botulinum D and F, VAMP 2 cleavage occurred concomitant with the impairment of caveolae-mediated cholera toxin B endocytosis. At the ultrastructural level, large, aberrant subplasmalemmal organelles were observed in treated cells, indicating that delivery of cholera toxin to intracellular intermediates (endosomes?) was impaired. Unfortunately, transcytosis was not examined in toxin-treated cells to determine whether VAMP 2 is a general regulator of caveolae-mediated internalization in endothelial cells. These findings also expand the function of VAMP 2 to include regulation of endocytic transport, whereas previously, this v-SNARE was thought to function in exocytic membrane docking and fusion. Whether VAMP 2 functions in PM vesicle docking and fusion in endothelial cells has not yet been tested. Likewise, a possible role for VAMP 2 in endocytosis in other cell types may warrant further investigation.

4. Munc18

Munc18 homologs have been identified in systems from yeast to neurons and are thought to participate in multiple vesicle transport steps (262, 442). The 68-kDa mammalian Munc18 proteins peripherally associate with the PM through interactions with syntaxins; in vitro, they bind syntaxins 1, 2, and 3 with nanomolar affinity. Interestingly, Munc18 binding to syntaxins cannot occur when the syntaxins are bound to SNAP-25 proteins, suggesting that the different complexes form reciprocally. However, it is presently not known whether Munc18 isoforms play a positive or negative regulatory role in PM targeting. Mutational analysis of related proteins in yeast, Drosophila, and Caenorhabditis elegans all implicate Munc18 species as positive regulators whereas in vitro assays suggest the opposite (262, 442). As for most of the SNARE molecules, no direct evidence for the involvement of Munc18 proteins in polarized PM targeting exists. However, the Munc18–2 isoform is primarily limited to polarized epithelial cells (472). Furthermore, its expression seems restricted to the apical PM where it forms complexes with syntaxin 3 (471), a characteristic that suggests a unique function for Munc18–2 in vesicle delivery to the apical PM.

5. The rab proteins

The rab proteins belong to the largest family of small molecular mass (20–30 kDa) GTP binding proteins. There are 11 known yeast isoforms and at least 60 rabs in
mammalian cells (195, 354, 500). Examination of transfected cells either overexpressing wild-type or dominant negative mutant (usually the GDP-bound conformer) forms of various rabs either stimulate or inhibit protein transport and in some cases alter organelle morphology. Although their precise roles are not known, they have been proposed to function in one of three ways: 1) facilitating vectorial traffic via associations with the cytoskeleton; 2) regulating vesicle docking by recruiting effector molecules, thereby promoting the formation of “molecular tethers”; and 3) “priming” docking and fusion by activating SNARE molecules (195, 500).

Given the large number of mammalian rabs and their varied distributions, it is likely that transcytosis in epithelial cells is regulated by multiple isoforms, but which ones? Rabs 3B, 13, 17, 18, 20, and 25 are preferentially expressed in epithelial cells (see Table 5), suggesting a unique function in polarized membrane transport. Although also expressed in nonpolarized cells, rabs 1, 2, 3D, 4, 5, 6, and 11 have also been implicated in regulating polarized PM transport. Of these 13 rabs, 9 have been localized to the apical pole: at the apical PM (rab3D), the tight junction (rabs 3B and 13), or in subapical structures (rabs 5, 11, 17, 18, 20, and 25). The multiple rab proteins in the apical region may point to the complexity of membrane transport events at this PM domain both in terms of specific transport steps as well as organellar intermediates.

Rab5 is the most extensively studied isoform, and much is known about the relationship between its catalytic activity and function in membrane transport (478). In all nonpolarized cells examined to date, rab5 is localized to the PM, clathrin-coated vesicles, and/or early endosomes. Overexpression of rab5 increased endocytic transport and stimulated early endosome fusion in vitro, whereas inhibition of rab5 led to the opposite effects. Recently, it was proposed that rab5 recruits EEA1 (one of its many effectors) to sites of endosome fusion along with NSF and syntaxin 13 that together drive formation of a large multimeric complex which then coordinates fusion pore assembly (368). This general role of rab5 in endocytosis strongly suggests an important role in the early steps of the transcytotic pathway in most polarized cells. Consistent with this is the presence of rab5a at the apical and basolateral endosomes in hepatocytes, hepatic WIF-B cells, in mouse kidney epithelia and MDCK cells (67, 272, 583). Furthermore, when overexpressed in MDCK cells, increased rates of fluid-phase internalization from the apical and basolateral PM were observed, suggesting a role for rab5a in endocytosis from both domains (67). In endothelial cells, rab5 is also localized to the PM and early endosomes, suggesting a role in endocytosis. However, it is not found associated with caveolae, suggesting it is not required for endothelial transcytosis (509).

Rab17 has been strongly implicated in regulating transcytosis in a number of different epithelial cells, first because of its restricted expression pattern and subcellular location, and more recently from functional studies. In kidney, rab17 expression was induced only upon mesenchymal differentiation to polarized cells (337). In intestinal tissue sections, it was detected only in polarized cells and not in surrounding, nonpolarized cells. In enterocytes, rab17 was detected in the basolateral domain, whereas in kidney cells it was found both at the basolateral PM and in apical tubules underlying the brush border (337). In MDCK and polarized Eph 4 cells, it was detected in subapically located vesicular structures (247, 631), and in hepatocytes, rab17 copuriﬁed with transcytotic vesicles (210). Functionally, the overexpression of wild-type rab17 in MDCK cells impaired the basolateral to apical transcytosis of diGα (247). Conversely, in Eph 4 cells expressing GTPase-deﬁcient mutants, the basolateral to apical transcytosis of Tf-R and a chimeric receptor was enhanced as was apical recycling of the chimeric receptor (631). Rab17 has also been copuriﬁed with a population of transcytotic vesicles from rat liver, suggesting it is an important regulator of hepatic basolateral to apical transcytosis, too. Surprisingly, both rab1 and -2 also copuriﬁed with the vesicles, implicating them as additional regulators of transcytosis (265). The next steps will be pinpointing the site of function in the transcytotic pathway, examining whether the role of rab17 is universal among epithelial cells and to identify the cellular effectors that rab17 activity regulates.

Rabs 11 and 25 have also been identiﬁed as important regulators of basolateral to apical transcytosis from functional studies performed in gastric parietal and MDCK cells (82, 134, 610). Both of these rabs have been localized to the apical recycling endosome in MDCK cells (82), and when the respective GTP-binding mutants were overexpressed, basolateral to apical transcytosing IgA accumulated in these structures (610). Interestingly, the activated form of rab25 inhibited transport more than the rab11 mutant. Also interesting is the observation that unlike for nonpolarized cells, rab11 is not required for Tf recycling (610). The puzzle is why these rabs apparently regulate the same transport steps at the apical PM. Do the differential responses suggest separable roles in transport? Also, why is rab11 required for Tf recycling in nonpolarized cells, but not polarized cells? Although rab25 expression is enriched in epithelial tissues, it is surprisingly absent in liver (193), pointing out yet another important difference in apical PM targeting in polarized hepatocytes.

The recent identiﬁcation of two rab11 effectors, rip11 and myosin Vb, has further conﬁrmed a role for this rab in regulating late steps in basolateral to apical transcytosis (304, 458). In both cases, dominant negative mutations signiﬁcantly impaired IgA apical PM delivery, and corresponding accumulations of IgA in the apical recycling endosome were observed in MDCK cells. Like rab11, my-
osin Vb was found to regulate Tf recycling only in nonpolarized cells (304) and rip11 mutants did not impair basolateral transferrin recycling (458). Are different rab11 effectors required for transferrin recycling in nonpolarized cells? How are these two effectors both regulated by rab11 at the same transport step? Careful dissection of the molecular events required for vesicle budding from the apical recycling endosome, transport to the apical PM, and subsequent docking and fusion are required to specifically identify how these molecules function.

The expression patterns of rabs13 and 3B are also highly dependent on the polarized state of a cell (611, 632). In nonpolarized cells, they were found in cytoplasmic vesicles, whereas in polarized cells, they were recruited to tight junctions. Rab13 has so far been detected at tight junctions of Caco-2 cells, mouse intestinal cells, kidney, and liver (632) while rab3B has been observed in tight junctions of colonic epithelia, kidney, and liver (611). In both cases, their localizations were dependent on the integrity of the tight junction. When junctions were disassembled by Ca^{2+} withdrawal, staining of rabs13 and 3B at the cell surface was lost, suggesting these rabs function in vesicle delivery to the tight junction and, in particular, regulate vesicular delivery of junctional components. At present whether any transcytotic vesicles are also specifically delivered to the cell surface at sites of cell-cell contact, and by extension, under the control of these rab isoforms, is not yet known. Immunoadsorption and examination of the vesicles with which these rab isoforms are associating will provide important clues to their function.

Interestingly, in a recent report (595) overexpressed, myc-tagged rab3B was localized not to tight junctions in MDCK cells, but to apically located structures that also contained unoccupied plgA-R. At present, there are no explanations for this different staining pattern. Nonetheless, in the presence of dlgA, rab3B dissociated from plgA-R and the GTP-bound mutant rab3B impaired dlgA-activated transcytosis to approximately control (-dlgA) levels. Together these results suggest that rab3B is a negative regulator of ligand-stimulated transcytosis in MDCK cells. It will be interesting to see if rab3B also regulates other forms of ligand-activated transcytosis in other cell types and whether the sites of delivery are at or near the tight junction.

Because of the congestion of different rab isoforms in the apical and subapical region of the polarized epithelial cell, it is important to identify the specific intermediates participating in basolateral-to-apical transcytosis. How many different subapical compartments exist, and of those, which receive transcytosing molecules? How do the rabs distribute among them? Furthermore, much of the morphological and functional analysis on the different rab isoforms has been performed in transfected cells overexpressing either wild-type or mutated proteins. Although these studies provided insight into rab function, careful enumeration and examination of the endogenous rab isoforms in a single cell type is required to clearly understand their role in vivo. Nonetheless, the concentration of rab proteins in the subapical regions of epithelial cells is striking and may point to the complexity of intracellular compartments and membrane transport events at this PM domain. It remains to be determined whether other putative transport machinery molecules or rab effectors are also concentrated at the apical regions of cells.

6. The exocyst

Over a dozen genes have been identified in yeast that are required for TGN to PM transport (275, 408, 409), and of these, more than two-thirds of the gene products form a multimeric complex referred to as the exocyst. The exocyst subunits were localized to the yeast PM, but only to sites of rapid cell growth at small bud tips (573). Interestingly, this expression pattern differs from the yeast t-SNARE molecules, which are evenly distributed at the PM. From these data, it was suggested that the exocyst mediates vesicle delivery to restricted regions of the cell surface such that it additionally discriminates (beyond SNARE function) whether and/or where a vesicle docks (573).

Mammalian homologs to the exocyst subunits are ubiquitously expressed and also form a multimeric complex that is mainly peripherally associated with the PM (239, 282, 574). The mammalian counterparts of the yeast Sec6, Sec8, and Sec10 exocyst have been localized to tight junctions in MDCK cells, and their discrete staining patterns were dependent on intact junctional complex formation (200, 324). Anti-rSec6 antibodies blocked transport of LDL-R from the TGN to the basolateral PM in permeabilized cells, whereas direct transport of an apical PM antigen, p75, was not changed (200). Conversely, overexpression of mammalian Sec10 enhanced PM transport of E-cadherin and basolateral secretion, whereas apical delivery of the integral PM protein gp135 was unchanged (324). Surprisingly, Sec10 overexpression also enhanced apical secretion. Taken together, these results implicates the exocyst as an important regulator of vesicle targeting to both PM domains, but delivery to the apical domain may be restricted to vesicles carrying secreted cargo. The placement of the complex further implicates the tight junction as an important site for vesicle delivery to either domain. Whether transcytotic vesicles are specifically recruited to these or other sites on the PM inhabited by the exocyst has not been rigorously examined, but remains an interesting possibility.

7. Annexins

Annexins are a large family of proteins grouped together due to shared amino acid sequence similarity and
their biochemical properties (103, 387, 563). One distinguishing feature of most annexins is their ability to aggregate membrane vesicles in the presence of Ca\(^{2+}\) in vitro (103). This activity led to the idea that annexins initiate membrane-membrane contact that results in fusion. Of the numerous annexin isoforms, a few have been identified as playing roles in polarized membrane targeting, but many of those were implicated based only on their subcellular location (see Table 5). However, more direct evidence has been demonstrated for annexins XIIIa and XIIIb, the former being an intestinal-specific isoform and the latter, a kidney-specific isoform (150, 618). In SLO-permeabilized MDCK cells, addition of either of these annexin isoforms enhanced direct apical PM delivery of HA (150, 309). Only the addition of recombinant XIIIa inhibited basolateral delivery of VSV-G. Accordingly, the addition of anti-annexin XIIIb antibodies specifically blocked transport of TGN-derived vesicles to the apical cell surface while transport to the basolateral surface was not changed (299). Unfortunately, the roles of annexin XIIIa and XIIIb in transcytosis were not tested in these studies. Some evidence for a role for annexin II in transcytosis comes from studies performed in isolated hepatocyte couplets (620). Upon induction of transcytosis by the addition of bile salts, annexin II immunofluorescence increased dramatically and was redistributed sequentially from beneath the basolateral PM to perinuclear structures and finally to the apical pole (620). Whether the annexin was simply a passenger on these transcytotic intermediates or was mediating transport activity is not known.

8. Dynamin

Dynamins are a family of high molecular mass (100 kDa), peripheral membrane-associated GTPases that function in the early stages of endocytosis (502, 586). Examination of dynamin function in transfected or microinjected mammalian cells has indicated its participation in clathrin-mediated endocytosis (106, 568) and suggested a role for dynamin in mediating fission of caveolae from the PM (223, 416). A role for dynamin in transcytosis has best been established in endothelial cells. Not only was dynamin found associated with caveolae in these cells, but it was also identified as a required factor for the release of caveolae from endothelial PM preparations in vitro (416). Caveolae-mediated internalization of cholera toxin B was also impaired in cultured endothelial cells expressing dynamin dominant negative constructs (416). Although these results imply that dynamin is a general regulator of caveolae-mediated internalization in endothelial cells, the effects of the dominant negative dynamins on transcytosing proteins were not analyzed.

Examination of dynamin isoforms in other polarized mammalian epithelial cells has been limited to cultured MDCK cells. Dynamin-2 was found at both PM domains and corresponding defects in IgA and Tf internalization from both domains were observed in cells expressing dominant negative dynamin mutants (7). Interestingly, dynamin-1 (the brain-specific isoform) localized only to the apical PM, and when the dominant negative dynamin-1 was expressed, internalization was inhibited only from that domain (7). Whether this is a physiologically relevant finding is not yet clear but may point to similarities in PM dynamics at the apical domain in epithelial cells and at the synapse in neurons. Nonetheless, many transcytosing receptors (including pIgA-R) are internalized via clathrin-coated vesicles, a process requiring dynamin activity. The prediction is that dynamin is required for the internalization of transcytosing molecules in polarized epithelial cells. Does dynamin regulate internalization of all transcytosing molecules, through caveolae, clathrin-coated, or noncoated endocytic vesicles, and at both PM domains?

B. Cytoskeleton

1. Microtubules and microtubule-based motors

In addition to the asymmetric distribution of PM proteins, the polarity of epithelial cells is also reflected in the organization of the cytoskeleton. In nonpolarized cells, microtubules emanate from a juxtanuclear microtubule organizing center (MTOC). In polarized cells, there is accumulating evidence that microtubules are instead (or additionally?) organized from sites at or near the apical PM such that the emanating microtubules are oriented with their minus ends at the apical PM and their plus ends attached to or near the basolateral PM (22, 131, 374, 475). Such an arrangement also dictates the placement of organelles within the interior of the epithelial cell (410, 434). In particular, in many epithelial cells, the compartments of the transcytotic pathway are linearly situated along the parallel microtubules. However, this arrangement is not universal to all polarized cells. For example, the microtubules in endothelial cells are arranged parallel, perpendicular, and obliquely to the long axis and in some cases even form criss-crossed helical arrays (53, 481).

In general, disruption of microtubules does not inhibit internalization of molecules (either soluble of membrane-associated) into early endosomal compartments, but does impair their movement to other compartments. This is true for molecules destined for transcytosis and internalized from the basolateral side in epithelial cells where the transcellular path is long, such as in colchicine- or nocodazole-treated MDCK cells, isolated hepatocytes or Caco-2 cells (56, 110, 137, 190, 222, 246, 346, 362). Interestingly, addition of colchicine impaired transcytotic delivery of albumin in endothelial cells in situ despite the comparatively short distance between the apical and basolateral surfaces (13). However, addition of microtubule
disrupting agents does not impair transcytosis of molecules internalized from the apical surface in MDCK or Caco-2 cells (246, 362). The reasons for this differential dependence on microtubules to the two domains are not yet understood. One possibility is that basolaterally destined vesicles have a shorter path to the lateral surface and thus a higher probability of encountering their target by simple diffusion. Alternatively, the machinery associated with basolaterally destined vesicles may promote more efficient and specific binding interactions with their target.

Microtubules are probably not a direct requirement for transcytosis; they likely facilitate delivery by providing the tracks upon which vesicles are translocated (43). Thus, when microtubules are disrupted, the kinetics of delivery are slowed, i.e., it takes a vesicle longer to encounter its appropriate target membrane by diffusion than when tracked along microtubules. However, this passive role for microtubules does not account for the mistargeting observed for some transcytosing molecules upon microtubule disruption. For example, the apical PM proteins, aminopeptidase N, DPP IV, and alkaline phosphatase, lost their polarized expression patterns in Caco-2 cells treated with nocodazole or colchicine (56, 137, 190). One explanation for the mistargeting is that different vesicle populations require the activities of distinct assemblies of docking and fusion molecules that differ in their binding specificities. Those vesicles with more promiscuous docking capabilities are able to associate with the improper domain upon microtubule disruption (e.g., apically targeted vesicles) resulting in apparent missorting. Alternatively, the mistargeting may reflect normal basolateral delivery of these PM proteins and the subsequent need for microtubules to facilitate transport to the apical PM domain.

The microtubule-based motor molecule that has received the most attention as a possible regulator of transcytosis is cytoplasmic dynein. In vitro analysis indicated that this megadalton, multisubunit molecule translocates vesicles in an ATP-dependent manner toward microtubule minus ends and that its activity is enhanced by another megadalton, multisubunit molecule, dynactin (279, 373). Since this endogenous dynein light chain Tctex-1 directly binds transfected rhodopsin in MDCK cells. When RP3, a non-rhodopsin-binding Tctex-1 homolog, was over-expressed in MDCK cells, it displaced the endogenous Tctex-1 in the dynein complex and disrupted apical delivery of rhodopsin (567). Interestingly, the apical distributions of HA and gp135 were not changed in these cells, nor were any basolateral antigens, suggesting that distinct light chains might regulate vesicle translocation. In permeabilized MDCK cells, when cytoplasmic dynein activity was abolished by either ultraviolet/vanadate photocleavage or immunodepletion from cytosolic extracts, direct apical delivery was impaired (298). On the other hand, immunodepletion of kinesin, a plus-end directed microtubule motor, inhibited transport to both the basolateral (to the plus ends as expected) and the apical (to the minus ends which was unexpected) domains. One possible explanation for this last result is that kinesin may be required to translocate vesicles through the microtubule meshwork that is postulated to exist between the Golgi and apical PM in MDCK cells (22). Finally, a surprising finding is that apical-organized microtubules were not required for transport from the TGN to either cell surface domain in MDCK cells (199). This implies that microtubules of both polarities, and by extension, both motor proteins facilitate transport to either PM domain, a report that contradicts numerous reports. Such continued confusion highlights the need for more information from multiple systems before we can confidently assign specific and/or generalizable roles for microtubules and their motors in transcytosis.

2. Actin and actin-based motors

The actin cytoskeleton also has a unique organization in many polarized cells. In general, actin microfilaments extend to the basolateral PM and form attachments through interactions with proteins of zonulae adherens, tight junctions, and focal adhesions. At the apical surface, actin is found as the core filament of microvilli and also as a dense subcortical web (58, 146, 366). At the basolateral domain, the actin-associated proteins fodrin and ankyrin form a scaffold that restricts the movements of certain integral PM proteins, including the Na+/K+/ATPase, thereby stabilizing the basolateral population (208, 366, 386). At the apical surface, actin was shown to be an important factor in maintaining the apical distribution of gp135, a membrane glycoprotein in MDCK cells, even in cells not in contact with their neighbors (419).

In general, actin is thought to be an important regulator of endocytosis from the apical, but not basolateral, PM (16). In MDCK, Caco-2, and pancreatic acinar cells,
addition of the actin disrupting agent cytochalasin D impaired internalization only from the apical domain (197, 261, 528). Clathrin-coated pits accumulated at the apical PM in each cell type, suggesting a block in clathrin-mediated internalization. In treated Caco-2 cells, apical internalization of folate was also decreased, implying that caveolea-mediated internalization was altered, and in MDCK cells, apical uptake of Lucifer yellow, which is internalized via noncoated vesicles, was decreased (197, 261). Thus actin may be important in many modes of apical internalization. However, there are exceptions to this generalization, as it was observed that the receptor-mediated internalization of ligand from the basolateral domain of hepatocytes was impaired by cytochalasin B treatment (280). Furthermore, treatment of Caco-2 cells with latrunculin B, another actin-disrupting agent, misdirected basolaterally internalized Tf-R and LDL-R into the apically directed transcytotic pathway (136). Limited evidence from studies in MDCK cells suggests that actin is also involved in facilitating steps in postendocytic transcytotic trafficking. In the presence of cytochalasin D, the delivery of transcytosing IgA from the basolateral early endosome to the apical recycling endosome was impaired 45% (346). Interestingly, IgA transcytosis was completely blocked when both cytochalasin D and nocodazole were added, suggesting that microfilaments and microtubules work in concert to facilitate transport to the apical cell surface (16, 346).

In support of the evidence indicating a role for actin in regulating membrane dynamics, recent studies have also implicated specific actin-based motors as important players in polarized vesicle trafficking. In particular, many classes of myosin motors have been localized to the subcortical actin network in many different epithelial cell types. The single-headed, short-tailed myosin I isoform has been localized to the apical brush border of intestinal (440) and kidney cells (94). They have also been found in association with zymogen granules at the apical aspect of pancreatic acinar cells (452) and in hepatocytes (25, 95). Also present at the intestinal brush border, although much less abundant, are the related myosin isoforms V and VI (218). Myosin VI has also been placed at the apical brush border of the proximal tubule cell line LLC-PK1 (215), whereas myosin Va has been also localized to subapical structures in polarized hepatic WIF-B cells (321) and myosin Vb at the apical recycling endosome in MDCK cells.

Functional studies on two different myosin isoforms have also placed them as potential regulators of transcytosis. Brush-border myosin I (BBM1) plays a role in postendocytic traffic at the basolateral pole, whereas myosin Vb is functioning at the apical pole (136, 304, 315). RhoA and rac1, two small GTPases that regulate actin cytoskeletal dynamics, also have also been implicated as regulators of polarized membrane transport with rhoA functioning at the basolateral surface and rac1 at the apical PM (270, 315). In particular, basolateral-to-apical transcytosing IgA accumulated in basolateral early endosomes in MDCK cells expressing inactivated forms of rhoA, whereas it accumulated in apical early endosomes in cells expressing dominant negative forms of myosin Vb or a constitutively activated form of rac1 (270, 304, 315). The mechanisms by which these motors, GTPases, and actin regulate membrane transport in polarized epithelial cells are not yet known but are the subject of a recent review (16).

C. Lipids and Transcytosis

The identification of proteins as important regulators of membrane transport is widely accepted and nearly indisputable. In the last several years, it has become more accepted that lipids also play significant roles in membrane transport. In particular, phosphoinositides, PC, cholesterol, and glycosphingolipids have been shown to be important players. Here we will focus on the roles of phosphoinositide 3-phosphate [PI(3)P], cholesterol, and glycosphingolipids in polarized membrane transport. Other lipids and their modifying enzymes have been the subject of many recent reviews (99, 242, 353).

1. PI(3)P

In the past several years, the role PI(3)P lipids play in regulating membrane transport has received considerable attention. This interest arose from early studies examining the effects of the selective phosphoinositide 3-kinase (PI 3-kinase) inhibitors wortmannin and LY294002 on membrane trafficking (98, 524, 584). To date, many transport pathways, including transcytosis, have been shown to be wortmannin and/or LY294002 sensitive. In MDCK cells, wortmannin treatment impaired basolateral to apical IgA transcytosis (78, 212). Likewise, both wortmannin and LY294002 disrupted transcytosis of pIgA-R and three newly synthesized resident apical PM proteins to the WIF-B apical domain (582) while wortmannin perfusion in isolated rat livers decreased the biliary release of basolaterally internalized HRP (156). Both agents were also observed to impair transcytosis in both directions of ricin in FRT cells and of neonatal FcR in IMCD cells (212, 370). Although the transcytosing proteins in treated WIF-B cells eventually reached their final destination (the apical PM), they transiently accumulated in basolateral early endosomes, indicating a block early in the transcytotic pathway (583).

Mammalian cells encode at least three different classes of PI 3-kinase isoforms (23, 162). Class I includes the p85/p110 heterodimeric kinases which consist of an 110-kDa catalytic subunit associated with a regulatory 85-kDa subunit. The other member is PI 3-kinase-γ, whose
catalytic activity is regulated by the βγ-subunits of heterotrimeric G proteins (80, 162, 591a). Class II PI 3-kinases include higher molecular weight kinases that contain C2 domains and class III kinases share the highest sequence similarity with the sole isoform identified in yeast, Vps34p (224, 604). The last kinase is also under the control of a regulatory subunit, p150 (in mammalian cells) or Vps15p (in yeast) (225, 427). All mammalian PI 3-kinase isoforms are sensitive to wortmannin and LY294002 (class II kinases at higher concentrations), which has led to ambiguity in distinguishing the roles that specific PI 3-kinases play in membrane transport. However, more recent studies that examined the effects of microinjection of specific inhibitory reagents on membrane transport have helped to begin assigning specific roles to specific kinase (529).

Using these inhibitory reagents in polarized hepatic cells, we found that specific inhibition of the class III PI 3-kinase Vps34p led to the formation of prelysosomal vacuoles containing endocytosed resident apical PM proteins and to the transient accumulation of transcytosing apical proteins in basolateral early endosomes (583). These results indicate that the lipid product of Vps34p, PI(3)P, regulates the two endocytic pathways differentially, at an early endosomal stage from the basolateral surface and from prelysosomes to lysosomes from the apical surface. The current model of PI(3)P’s role in endocytosis invokes recruitment of Vps34p/p150 by activated rab5 to the sites of endosome-endosome fusion and local production of PI(3)P (98, 559, 625). The PI(3)P-binding protein early endosomal antigen 1 (EEA1) is recruited to these sites where PI(3)P and rab5 binding stabilize its membrane association. The stabilized EEA1 molecules then form oligomers that coordinate the formation of a large vesicle docking site also containing NSF and syntaxin13, all of which drive endosome fusion. Thus, in nonpolarized cells, when PI(3)P lipids were depleted by wortmannin, EEA1 association with early endosomes was lost and the subsequent events were affected. Likewise, we found that EEA1 dissociated from basolateral early endosomes in treated WIF-B cells; concomitantly, we observed delayed basolateral to apical transcytosis. All of these results are consistent with the current model. In contrast, the block we observed at a late endocytic step in the apical pathway is not consistent with the existing model (583). Disruption of EEA1 function in the fusion of early endosomes arising from the apical surface should have blocked an earlier step in the pathway. However, a subpopulation of EEA1 near the apical surface remained membrane-bound under PI(3)P-depleting conditions, suggesting that the protein remained active and allowed progression of endocytosed proteins along the apical route thereby revealing a block downstream.

Injection of p110α inhibitory antibodies into WIF-B cells was also found to impair basolateral to apical transcytosis as did anti-Vps34p injection (583). However, increased basolateral surface staining of the transcytosing markers was observed with no corresponding intracellular accumulations, suggesting that Vps34p and p110α act at separate steps of the pathway, with p110α possibly acting at internalization. Together, these results lead to a number of questions. Are the apical and basolateral pathways in other polarized epithelial cells also differentially regulated by PI(3)P? Are the functions of p110α and Vps34p in basolateral-to-apical transcytosis conserved among other epithelial cells? What PI(3)P-binding proteins are required for membrane transport at the distinct steps?

2. Cholesterol and glycosphingolipids

Glycosphingolipids and cholesterol are enriched in cell surface membranes in all eukaryotic cells. In polarized epithelial cells, the apical surface is even further enriched for these lipid species (158, 281, 295, 597). One possible function of cholesterol and glycosphingolipids is to impart structural rigidity and decreased permeability to the apical domain, which in turn protects the cell against the harsh external environment it faces (e.g., the detergent-like bile or high acidity) (295). The differences in these environments would therefore dictate the lipid compositions required for appropriate protection and function in different epithelial cell types. The intrinsic properties of glycosphingolipids and cholesterol promote their assembly into specialized membrane domains called “rafts” (63, 214). Within these cell surface domains are selected proteins that are recruited based on their physical properties. In particular, GPI-anchored proteins that are predominantly expressed at the apical surfaces of epithelial cells localize to rafts.

These observations in combination with studies performed mainly in MDCK cells have led to the “raft” hypothesis for protein sorting. According to this hypothesis, rafts form in the biosynthetic pathway where they recruit apically destined proteins (especially GPI-linked proteins); the rafts with their recruited cargo are then transported directly to the apical domain in vesicles. There is considerable experimental evidence to support this hypothesis. For example, GPI anchors have been shown to be sufficient to target proteins to the apical domain (62, 325). Furthermore, rafts have been isolated based on their insolubility in nonionic detergents (especially Triton X-100) at 4°C, GPI-anchored proteins copurify with them (297), and cholesterol-depleting drugs and sphingolipid synthesis inhibitors disrupt delivery of apical PM residents (252, 297). Recent work has also suggested that caveolins and caveolae-like vesicles are important for PM delivery of GPI-anchored proteins. In caveolin-1 or caveolin-3 knock-out mice, GPI-anchored proteins were re-
tained at the TGN in mouse embryo fibroblasts or muscle tissue, respectively (551).

However, there are many observations that are inconsistent with the raft hypothesis of sorting (612). In FRT cells, both glycosphingolipids and GPI-anchored proteins are sorted to the basolateral domain, whereas in certain MDCK strains, they are evenly distributed between the two domains. However, in both cases, other apical PM proteins are sorted properly to the apical surface. In hepatocytes, GPI-anchored proteins, such as 5′-nucleotidase, are first transported to the basolateral domain before apical delivery (499). Furthermore, many nonapical proteins have also been detected in purified raft fractions.

Despite the debate, the question still remains. Do rafts sort apically destined proteins in the transcytotic pathway? If so, are rafts present at the basolateral domain or in other transcytotic intermediates? Two approaches have been taken to begin answering these questions. First is examining whether transcytosing proteins are present in detergent-insoluble fractions and second is whether they are internalized from the cell surface via caveolae, specialized raft domains. So far, the first approach has yielded conflicting results from studies performed in enterocytes as well as FRT and MDCK cells (210, 493). In polarized enterocytes from mouse intestinal explants, a significant proportion of basolateral to apical transcytosing IgA (secreted from neighboring mucosal plasma cells) was found in detergent-insoluble rafts (210). Because IgA is internalized and delivered to the apical surface via the pIgA-R, the data imply that the receptor must also be raft associated. Accordingly, ~50% of the pIgA-R was recovered in detergent-insoluble fractions (210). This result contradicts that reported for pIgA-R in MDCK and FRT cells; pIgA-R was not found in Triton X-100-insoluble fractions at any point during its life cycle (493). The reasons for these opposing observations are likely not due to differences in raft preparation, since the methods used were very similar. Instead, the differences may be related to the intrinsic differences between cell types, between in vitro versus in vivo systems, or between endogenously (in enterocytes) or exogenously (in FRT and MDCK cells) expressed molecules. None of these possibilities has yet been well explored.

The second approach to determine whether rafts sort apically destined proteins in the transcytotic pathway has focused on examining interactions of transcytosing proteins with caveolae. Because not all rafts are associated with caveolin, caveolae have more recently been classified as specialized rafts. Much of what we know about caveolae in polarized epithelial cells comes from studies in endothelial cells where these structures are highly abundant (see sect. iv). Otherwise, caveolae have been examined in only a limited number of polarized cells. Despite the enrichment of cholesterol and glycosphingolipids at the apical domain, caveolae have only been observed at the basolateral domain of MDCK cells and in kidney epithelial cells in situ (57, 435, 498). Like endothelial cells, MDCK caveolae formation is dependent on cholesterol levels (205, 267, 511). In both cases, no morphologically definable caveolae were observed in cells treated with cholesterol-depleting drugs such as filipin or cyclo-dextrin. In endothelial cells, the cholesterol-dependent loss of caveolae corresponded to decreases in albumin transcytosis both in vitro and in vivo (267, 511). When the drugs were withdrawn and cholesterol synthesis stimulated by the addition of mevalonate in MDCK cells, or the addition of 10–20% serum in endothelial cells, the caveolae reformed (205, 511). FRT and Caco-2 cells express little to no detectable caveolin, and no caveolae have been observed (322, 603). However, when caveolin-1 was over-expressed in these cells, caveolae were observed in both domains in FRT cells and only at the basolateral domain of Caco-2 cells (322, 603). Interestingly, the formation of caveolae in FRT cells did not promote apical sorting of GPI-anchored proteins or their sorting into rafts (322), suggesting that rafts are not responsible for apical targeting in these cells. Alternatively, other components may be lacking in FRT cells that are necessary for sorting into rafts and/or subsequent apical delivery.

Our challenge is to begin carefully examining the detergent solubility properties of multiple endogenous apical PM protein types (e.g., GPI-linked, single transmembrane, polytopic) during their entire life cycles within one cell type to determine the role of rafts in transcytosis. The detergents used should not be limited to Triton X-100, as it has been recently reported that in PC12 cells, subpopulations of rafts exist with different solubility properties (482). The effects of both cholesterol and glycosphingolipid disruptors on transcytosis should be directly tested. Also, examination of purified organellar intermediates for raft components and protein insolubility properties may help clarify at which step(s) rafts are required. Such a strategy has shown that recycling endosomes (immunoisolated with anti-Tf-R antibodies) are enriched for raft components including glycosphingolipids, cholesterol, caveolin, and another raft-associated protein, flotillin (167). Such careful and consistent experimentation will more clearly determine the role of rafts in apical vesicle targeting. Recent evidence also suggests that the PM-associated t-SNAREs are organized in cholesterol-dependent surface domains in nonpolarized cells (87, 303). Are these domains required for vesicle delivery to the apical PM?

D. Perturbations of Transcytosis

The use of perturbants has long been a way to begin dissecting the mechanisms and molecules involved in
regulating complex cellular processes. Chemical perturbers are commonly used and have provided insight into understanding vesicle transport in mammalian cells. Of particular note are alkylating agents (e.g., NEM), drugs that specifically disrupt the cytoskeletal systems of the cell and specific lipid kinase inhibitors which were discussed in section V, A–C. In this section, we focus on the perturbation of acute regulation of vesicle transport by agents that alter the functions of heterotrimeric G proteins, intracellular calcium (Ca$_i$) homeostasis, protein kinase A (PKA), or protein kinase C (PKC) activity (Table 6).

1. Heterotrimeric G proteins and PKA

Accumulating evidence supports a role for heterotrimeric G proteins in regulating vesicle transport in both the endocytic and exocytic pathways (4, 41, 42, 220, 412, 445, 561). Examination of the effects of various agents on epithelial cells, especially MDCK cells, have indicated that transcytosis is also regulated by G proteins. The first clues came from treating cells with nonselective G protein activators, guanosine 5’-O-(3-thiotriphosphate) (GTPyS) and AlF. In both cases, transcytosis to the apical, but not basolateral, domain was slightly enhanced (42). Addition of specific G protein ADP-ribosylating toxins (cholera or pertussis toxins) further indicated that transport to the apical domain (from both the TGN and via transcytosis) is regulated by the Gs $\alpha$-subunit (31, 42, 211). Mastoparan, a G$_\gamma$-activating peptide had no effect on pIgA-R apical delivery while anti-Gs $\alpha$-antibodies were slightly inhibitory, confirming a role for Gs in transcytosis to the apical domain (42). In intact cells, the overexpression of the wild-type or constitutively active Gs $\alpha$-subunit led to a modest increase in transcytosis (211). Interestingly, the addition of either the Gs $\alpha$- or $\beta\gamma$-subunits to an in vitro system led to a small increase in the formation of transcytotic vesicles (42). However, it is not known whether the $\beta\gamma$-subunits were acting to inhibit another G protein that negatively regulates transcytosis or in concert with the $\alpha$-subunit.

One well-studied result of Gs $\alpha$ activation is the activation of adenyl cyclase and the subsequent increased production of cAMP. PKA is then activated by the increased cAMP levels, which puts in motion numerous (but not well-defined) cellular processes. To determine whether this cascade of events is involved in vesicle transport, another handful of chemical perturbants has been useful. In particular, forskolin (a direct activator of adenyl cyclase) has been shown to enhance transcytosis to the apical, but not basolateral, domain in MDCK cells (211). This effect was also seen by the addition of exogenous cAMP to both MDCK cells and intact rat hepatocytes (211, 217). A PKA inhibitor, H-89, produced the opposite effect, consistent with a role for PKA in regulating transcytosis (211). Some evidence suggests that G protein/PKA regulates transcytosis from apical endosomal compartments to the apical PM (31). Interestingly, this is also the site(s) at which at least nine small-molecular-weight GTP-binding proteins have been proposed to function, further exposing the complexity of vesicle transport events in this region of a polarized epithelial cell.

Trimeric G proteins are also important regulators of endothelial transcytosis, but in this case, Gs has been examined most extensively. In polarized endothelial cells in vitro, addition of pertussis toxin (a Gs$_i$ inhibitor) or expression of a dominant negative Gs$_{2}$ peptide inhibited apical to basolateral albumin transcytosis mediated by activated gp60 (379). Previously, treatment of endothelial cells with tyrosine kinase inhibitors suggested that a src-mediated signaling pathway regulated transcytosis (Ref. 576 and discussion in sect. V A3); thus Minshall and colleagues examined whether Gs and src signaling were coupled. Consistent with this hypothesis, overexpression of dominant negative src prevented the association of Gs$_{2}$ with caveolin (caveolae?) in caveolin-I-overexpressing cells. Furthermore, dominant negative src also inhibited albumin transcytosis. From these results, the authors suggested that gp60 activation recruits Gs$_{2}$ to caveolae that in turn sets off a src-mediated signaling cascade that activates transcytosis (379). This situation is somewhat analogous to the activation of p130-Gr transcytosis by ligand binding where another tyrosine kinase, p62$^{res}$, is involved (341). Thus tyrosine phosphorylation may be a common mechanism for regulating activated transcytotic pathways.

2. Calcium, calmodulin, and PKC

The use of another set of pharmacological agents has indicated that vesicle trafficking is also acutely regulated by changes in Ca$_i$ levels. Thapsigargin, a selective inhibitor of sarco/endoplasmic reticulum Ca$^{2+}$-ATPases, enhanced transcytosis to the apical domain in MDCK cells, whereas BAPTA, a Ca$^{2+}$-chelating compound, inhibited it (79). Neither agent altered transcytosis in the opposite direction. The mechanism whereby Ca$_i$ fluxes are manifested in changes in vesicle transport is not known, but likely includes alterations in Ca$^{2+}$-dependent enzyme activities. This has been substantiated by studies examining the effects of the calmodulin antagonists W-7, W-13, and trifluoperazine in MDCK cells (18, 244, 329). All three agents significantly impaired basolateral to apical transcytosis of dIgA while transcytosis in the same direction of the fluid-phase marker ricin was enhanced. In both cases, endocytosis from the basolateral surface was unchanged, suggesting that the agents were acting later in the pathway. Consistent with this is the finding that dIgA accumulated in large endosomal structures located in the apical region of cells treated with W-13 (18). Interestingly, apical
to basolateral transcytosis of ricin was not altered by these agents, yet an increase in its endocytosis from the apical domain was observed (329). However, megalin-mediated transcytosis of thyroglobulin in the same direction was inhibited in thyroid cells (FRTL-5) treated with W-7 and trifluoperazine (349). Thus CaM regulation is important for many steps in transcytosis. The challenge is to pinpoint the specific calmodulin-dependent enzymes that are functioning at these transport steps to understand the differential effects of these agents.

PKC has also received attention as a possible regulator of transcytosis based both on the effects of changing Ca_2+

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### TABLE 6. Effects of pharmacological agents on transcytosis

<table>
<thead>
<tr>
<th>Agent</th>
<th>Mode of Action</th>
<th>BL-A</th>
<th>A-BL</th>
<th>Epithelial Cell Types Examined</th>
<th>Comments</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEM</td>
<td>Alkylates sulfhydryls</td>
<td>N/D</td>
<td>MDCK and endothelial cells, hepatocytes</td>
<td>Very nonselective and will inhibit multiple ATPases</td>
<td>17, 253, 305, 564</td>
<td></td>
</tr>
<tr>
<td>Bacterial toxins</td>
<td>Proteolytically cleaves v- and t-SNAREs</td>
<td>↓ N/D</td>
<td>MDCK cells</td>
<td>Each toxin is highly selective for its target</td>
<td>17, 26, 27, 314</td>
<td></td>
</tr>
<tr>
<td>Wortmannin</td>
<td>PI 3-kinase inhibitor</td>
<td>↓ -/↓</td>
<td>MDCK, FRT, IMCD and WIF-B cells, hepatocytes</td>
<td>Class I and III kinases inhibited at low concentrations (nM), all classes inhibited at high concentrations (μM), irreversible</td>
<td>78, 156, 212, 370, 582</td>
<td></td>
</tr>
<tr>
<td>LY294002</td>
<td>PI 3-kinase inhibitor</td>
<td>↓ -/↑</td>
<td>IMCD and WIF-B cells</td>
<td>All classes inhibited at high concentrations (μM), reversible</td>
<td>370, 582</td>
<td></td>
</tr>
<tr>
<td>Filipin</td>
<td>Binds and removes cholesterol from PM</td>
<td>↓ N/D</td>
<td>Endothelial cells</td>
<td>Specific for unesterified cholesterol, reversible</td>
<td>509</td>
<td></td>
</tr>
<tr>
<td>Nocodazole</td>
<td>Microtubule depolymerizer</td>
<td>↓ -/↓</td>
<td>Many cell types</td>
<td>Specific, cell permeant, reversible</td>
<td>56, 110, 137, 222, 246, 346, 362, 370, 453, 548</td>
<td></td>
</tr>
<tr>
<td>Colchicine</td>
<td>Inhibits tubulin polymerization</td>
<td>↓ -/↓</td>
<td>Many cell types</td>
<td>Specific, irreversible</td>
<td>13, 390</td>
<td></td>
</tr>
<tr>
<td>UV/Vanadate</td>
<td>ATPase inhibitor</td>
<td>↓ -/↓</td>
<td>MDCK cells</td>
<td>Not readily cell permeant, potent inhibitor of many ATPases, especially ion pump ATPases</td>
<td>288</td>
<td></td>
</tr>
<tr>
<td>Cytochalasin D</td>
<td>Actin depolymerizer</td>
<td>↓ -/↓</td>
<td>Many cell types</td>
<td>Specific, cell permeant, reversible</td>
<td>197, 261, 346, 528</td>
<td></td>
</tr>
<tr>
<td>GTPγS</td>
<td>G protein activator</td>
<td>↑ N/D</td>
<td>MDCK cells</td>
<td>Activates all GTPases</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>AIF</td>
<td>G protein activator</td>
<td>↑ N/D</td>
<td>MDCK cells</td>
<td>Activates all heterotrimeric G proteins</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>Cholera toxin</td>
<td>ADP-ribosylates and activates Gα subunit</td>
<td>↑/↓ N/D</td>
<td>MDCK cells</td>
<td>Holotxin must bind Gαi to be internalized, A subunit (with ADP ribosylation activity) is not cell permeant</td>
<td>31, 41, 211, 379</td>
<td></td>
</tr>
<tr>
<td>Pertussis toxin</td>
<td>ADP-ribosylates and activates Gα subunits</td>
<td>– N/D</td>
<td>MDCK cells</td>
<td>Holotxin must bind cell surface to be internalized, A protem (with ADP ribosylation activity) is not cell permeant</td>
<td>31, 42, 211</td>
<td></td>
</tr>
<tr>
<td>Mastoparan</td>
<td>Gα, Gβ, and Gγ subunits</td>
<td>– N/D</td>
<td>MDCK cells</td>
<td>Not readily cell permeant</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>forskolin</td>
<td>Adenyl cyclase activator</td>
<td>–/↑</td>
<td>MDCK cells</td>
<td>Specific</td>
<td>211</td>
<td></td>
</tr>
<tr>
<td>cAMP</td>
<td>PKA activator</td>
<td>–/↑</td>
<td>MDCK cells and hepatocytes</td>
<td>Specific, cell permeant</td>
<td>211</td>
<td></td>
</tr>
<tr>
<td>H-89</td>
<td>PKA inhibitor</td>
<td>↓ –</td>
<td>MDCK cells</td>
<td>Potent, selective</td>
<td>211</td>
<td></td>
</tr>
<tr>
<td>Thapsigargin</td>
<td>Ca_2+ -ATPase inhibitor</td>
<td>↑ N/D</td>
<td>MDCK cells</td>
<td>Potent and specific for sarco endoplasmic reticulum Ca_2+ -ATPases</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>BAPTA</td>
<td>Calcium chelator</td>
<td>↓ –</td>
<td>MDCK cells</td>
<td>102-fold greater affinity for Ca_2+ than Mg_2+; available in a cell permeant form</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>W-7</td>
<td>CaM antagonist</td>
<td>↑/↓</td>
<td>MDCK cells and FRT and MDCK cells</td>
<td>Cell permeant</td>
<td>244, 329, 340</td>
<td></td>
</tr>
<tr>
<td>W-13</td>
<td>CaM antagonist</td>
<td>↓ N/D</td>
<td>MDCK cells</td>
<td>Cell permeant</td>
<td>18, 349</td>
<td></td>
</tr>
<tr>
<td>Trifluoperazine</td>
<td>CaM antagonist</td>
<td>↑/↓</td>
<td>MDCK cells and FRT and MDCK cells</td>
<td>Elevates intracellular Ca levels at low concentrations, antagonizes CaM at higher concentrations, cell permeant</td>
<td>329</td>
<td></td>
</tr>
<tr>
<td>KN-62</td>
<td>CaM kinase II inhibitor</td>
<td>– –</td>
<td>MDCK cells</td>
<td>Potent, selective</td>
<td>329</td>
<td></td>
</tr>
<tr>
<td>PMA</td>
<td>PKC activator</td>
<td>↑ N/D</td>
<td>MDCK cells</td>
<td>Cell permeant, potent</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>H-7</td>
<td>Serine/threonine kinase inhibitor</td>
<td>– N/D</td>
<td>MDCK cells</td>
<td>Nonselective, cell permeant</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Herbrimycin</td>
<td>Tyrosine kinase inhibitor</td>
<td>↓ N/D</td>
<td>Endothelial and MDCK cells</td>
<td>Highly selective for tyrosine kinases, cell permeant, irreversible</td>
<td>340, 576</td>
<td></td>
</tr>
<tr>
<td>Genestein</td>
<td>Tyrosine kinase inhibitor</td>
<td>↓ N/D</td>
<td>MDCK and endothelial cells</td>
<td>Nonselective</td>
<td>340, 576</td>
<td></td>
</tr>
<tr>
<td>PP1</td>
<td>Tyrosine kinase inhibitor</td>
<td>↓ N/D</td>
<td>MDCK cells</td>
<td>Selective for Src tyrosine kinases</td>
<td>340</td>
<td></td>
</tr>
</tbody>
</table>

↑, Increases; ↓, decreases; –, no change; CaM, calmodulin; IMCD, inner medullary collecting duct; N/D, not determined; NEM, N-ethylmaleimide; GTPγS, guanosine 5’-O-(3-thiotriphosphate); PKC, protein kinase C; PKA, protein kinase A; PI, phosphatidylinositol.
levels and on the use of phorbol esters, potent PKC activators. When one such phorbol ester, phorbol 12-myristate 13-acetate (PMA), was applied to MDCK cells, both apical recycling and basolateral to apical transcytosis of dIgA and transferrin were enhanced, suggesting PKC was acting at an apical recycling compartment (79). PMA treatment also led to the membrane recruitment of α and ε PKC isoforms (79). Interestingly, dIgA binding to its receptor (conditions that activate its own transcytosis) also activated PKC-ε, which led to increased levels of inositol 1,4,5-trisphosphate and Ca²⁺, the latter of which stimulated transcytosis (79, 548). The rise in Ca²⁺ is likely mediated via inositol 1,4,5-trisphosphate-sensitive intracellular stores; thus ligand binding initiates a signal that is propagated across the cell independent of the ligand-receptor complex itself (340). Paradoxically, treatment of MDCK cells with H-7, a specific PKC inhibitor, did not inhibit dIgA or ricin transcytosis in MDCK cells (18, 329). At present, there is no good explanation for these disparate results.

3. Possible mechanisms

In all the studies cited above, it is important to point out that in many cases, the inhibitory and stimulatory effects of transfection or addition of pharmacological agents on PM targeting were small. Does the size of the response in vitro reflect loss of normal regulation that would be observed in vivo or does it represent fine-tuning that may be critical for proper organ function? If it is the latter, what appears to be a minimal change in vitro may have a large impact on the overall homeostatic balance of the organism. Thus physiological studies are needed. Specifically, our challenge is to identify the molecules in membrane transport that are regulated by phosphorylation or calmodulin/Ca²⁺ binding. Few if any direct links have yet been established, but there are some examples of where the modifications of molecules implicated in transcytosis correlate with their proposed functions.

Many of the SNARE molecules are phosphorylated in vitro by purified kinases, and the modification alters their binding properties. In particular, α-SNAP is a substrate for PKA and when phosphorylated, its ability to bind the core docking and fusion complex was decreased 10-fold (230). In vitro, syntaxin 4 was shown to be phosphorylated by PKA, casein kinase II (CKII), and PKC, and this phosphorylation disrupted its binding to SNAP23 or SNAP25 (92, 159, 474). When syntaxin 4 was used as bait in a yeast two-hybrid screen, a novel SNARE kinase (SNAK) was identified, but surprisingly, SNAP-23 was overwhelmingly its preferred substrate in vitro and in vivo (73). SNAK-phosphorylated SNAP-23 was not associated with the ternary complex, whereas phosphorylation of syntaxin 1 by CKII enhanced t-SNARE’s association with SNAP-25 (154). Interestingly, by using phosphospecific antibodies to stain neurons, it was found that the phosphorylated form of syntaxin 1 was localized to discrete regions along the axonal PM that did not colocalize with synaptic vesicles (154). Another SNARE hypothesis molecule, Munc18–1, is a substrate for PKC and cyclin-dependent kinase 5, and phosphorylation in this case inhibited binding to syntaxin 1 (123a, 164, 527).

Several rabs and rab effector proteins have also been shown to be phosphorylated. In vitro, GDI phosphorylation is mediated by PKA (555). In vivo, the phosphorylated GDI associated to the cytosolic form of rab5, while the unphosphorylated GDI was bound to the membrane-associated rab5. These data suggest that the cycling of rab proteins between donor and acceptor membranes is also a regulated process. The rab effector protein rabphilin 3A is phosphorylated by PKA in vitro (411). Another rab effector, rab8ip, is a serine/threonine protein kinase itself (GC kinase) that is activated by the stress response in lymphocytes (466). The phosphorylation state of rip11, a rab11 effector, may regulate its membrane binding properties. In polarized MDCK cells, conditions that decrease rip11 phosphorylation (e.g., staurosporine treatment) enhanced its binding to membranes (458). Caveolin, the major structural protein of caveolae, has been shown to be tyrosine phosphorylated in endothelial cells under conditions where transcytosis was stimulated (191, 318, 358). Both kinesin and cytoplasmic dynein are phosphorylated in vitro and in vivo, and this modification has been correlated with their ability to transport vesicles (including transcytotic vesicles?) along microtubules (320, 486, 494). The light chain of the actin based myosin I motor proteins is calmodulin, which is thought to regulate motor activity (449). Annexin binding to membrane lipids, and by extension, ability to promote intermembrane associations, is dependent on Ca²⁺ (103).

E. Transcytosis Versus Direct PM Delivery

Transcytosis is only one pathway that molecules take to a specific PM domain. Both newly synthesized PM proteins and secreted molecules can also be delivered directly from the TGN to either PM domain. How different are the mechanisms regulating vesicle transport along these pathways? As expected, transport directly from the TGN to either the apical or basolateral domains is regulated differently. In particular, differences in the involvement of SNARE molecules were observed in permeabilized MDCK cells (17, 253, 332). Addition of anti-NSF antibodies, NEM, mutant NSF, rab-GDI, or tetanus and botulinum F neurotoxins all inhibited basolateral targeting of VSV-G protein, whereas targeting from the TGN to the apical domain of HA was not changed (17, 253). This implies that basolateral targeting requires NSF, rab proteins, and VAMP 2. Conversely, syntaxin 3 overexpression or anti-syntaxin 3 antibodies inhibited only apical delivery.
Addition of α-SNAP antibodies and treatment of cells with botulinum E inhibited transport to both domains (17, 253), whereas TI-VAMP antibodies inhibited apical transport (the effects of theses antibodies on basolateral targeting were not examined) (300). Taken together, targeting to both domains requires SNARE molecules, but in different combinations. Surprisingly, apical targeting is NSF independent, suggesting the involvement of an as yet unidentified homolog. Microtubule and actin filament disruption also has differential effects on direct delivery to either domain in MDCK cells; apical delivery is inhibited, whereas, in most cases, basolateral is not (56, 137, 190, 362, 420, 598). In addition, PKA, PKC, and calmodulin-mediated mechanisms appear to acutely regulate delivery mainly to the apical domain (79, 329, 447).

How different are the targeting mechanisms regulating transcytosis? This question has so far been best addressed in studies performed in MDCK cells. Unlike TGN to apical delivery of HA-containing vesicles, pIgA-R-mediated transcytosis appears to require NSF activity (17, 253). Furthermore, syntaxin 3 (required for direct apical targeting) is not involved in mediating transcytosis of IgA (332), SNAP-23, on the other hand, is involved in both basolateral to apical transcytosis, and direct apical and basolateral targeting (332). Also, both direct and transcytotic delivery to the basolateral domain does not require microtubules (see sect. viii). Based on the effects of pertussis toxin on MDCK cells, transport from the TGN to the basolateral PM was found to involve the Gα subunit of heterotrimeric G proteins, whereas transcytosis to this domain was not (31, 211, 446). From these results (and others) it is clear that the mechanisms cells use to regulate cell surface delivery are complex and are dependent on factors that are not yet understood.

Another interesting twist to apical PM sorting has come from studies looking at the raft-associated protein referred to as MAL. This 17-kDa tetra-spanning TMD protein, first identified in myelin and lymphocytes (hence MAL), is also expressed in many epithelial cell types (30, 221). The ectopic expression of human MAL rescued the defect (120). In HepG2 cells treated with antisense MAL2 oligonucleotides, transcytosis of pIgA was impaired from early endosomes to a juxta-apical compartment. Thus different MAL isoforms may be responsible for specialized domain-specific protein sorting. This is further suggested by the finding that yet another MAL family member, BNE, is expressed in endothelial cells where it is associated with caveolae (119).

VI. CONCLUSION

We have learned a lot about transcytosis since its existence was first postulated over 50 years ago. We have identified cargo, uncovered pathways, and determined possible mechanisms. Nonetheless, many things remain mysterious. How does the cell discriminate between cargo destined for transcytosis versus degradation in lysosomes? In peripheral endothelial cells, it appears that separate entry points dictate different fates; internalization via coated pits sends cargo to the endocytic pathway, whereas caveolae-mediated internalization ensures a transcytotic fate. However, we need further study to make a definitive conclusion on this point. In other epithelial cells, the transcytotic pathway is a branch of the endocytic pathway. Where and how is transcytotic cargo sorted in these cells? Signals have been identified in the pIgA-R cytoplasmic tail that prevent its degradation. How are they recognized? Are they universal? In enterocytes, megalin recycles to the PM from endosomes after internalization and dissociation from its transcytotic cargo, whereas in thyroid, the same membrane receptor escorts thyroglobulin across the cell. What signals navigate megalin along these different itineraries and how do cells discriminate between the possible fates? Another unsolved problem is understanding how the cell determines what should be transcytosed versus that which should be diverted for its own use. Are the acute regulatory mechanisms described in section viii important here?

How might transcytosis dysfunction or corruption contribute to human disease? For example, do mistakes in self-apportionment lead to vitamin B12 deficiency? Do mistakes in cargo selection cause disease? In enterocytes, the transcytosis of undigested food antigens to underlying interstitial cells has been linked to food hypersensitivity and allergies (630). The antigenic epitopes stimulate the immune system leading to the production of cytokines that ultimately result in the loss of enterocyte barrier function and diarrhea. Similarly, certain pathogens co-opt the transcytotic pathway of M cells for infection. Recent evidence suggests that Streptococcus pneumoniae binds unoccupied, uncleaved pIgA-R that has been transcytosed to the apical surfaces of nasopharyngeal epithelial cells. The receptor-pneumonia complex is thought to be apically internalized and transcytosed to the basolateral surface where the pathogen is released.

Finally, how is transcytosis used or adapted in development? This is a huge unexplored area where there will undoubtedly be surprises. Let us hope that the next 50 years will provide us with some exciting answers.
We thank R. Fuchs, L. Ghitescu, M. Lisanti, M. Molliver, M. Neutra, S. C. Silverstein, and M. Wessling-Resnick for sharing their expertise in areas of transcytosis less familiar to us. Thanks also to the Editorial Board (Susan Hamilton) for their patience during the long gestation period before seeing this review.

Our research has been supported by National Institutes of Health Grants P01-DK-44375, R01-GM-29185, NRSA-DK-09620, and T32-DK-07632.

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