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# SIGNALS AND MECHANISMS OF SORTING IN EPITHELIAL POLARITY

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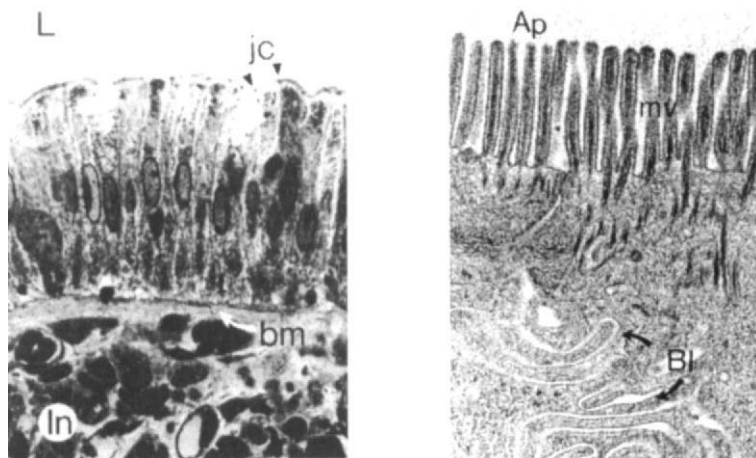
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## I. INTRODUCTION

Polarized epithelial cells have long captured the attention of cell biologists and cell physiologists. This is largely because the architecture of these cells so tellingly speaks their function. At the electron microscopic level, one of the most apparent and fundamental features of this cell type is its polarized organization of intracellular organelles and its structurally and compositionally distinct luminal (apical) and serosal (basolateral) plasma membrane domains (Figures 1A, B). Through the eyes of the physiologist, the polarized epithelial phenotype is an absolute necessity for organ system function. In the most general sense, these cells organize to form a continuous, single layer of cells, or epithelium, which serves as a semi-permeable barrier between apposing and biologically distinct compartments. Within the tubules of the nephron, these cells orchestrate complex ion-transporting processes that ultimately control the overall fluid balance of the organism. At the surface of the gastrointestinal tract, specialized versions of this cell type control the digestion, absorption and immuno-protection of the organism. Thus while polarized epithelial cells can carry out myriad functions, they share one defining feature: a structural polarity which serves their underlying functional polarity.

### A. Epithelial Membrane Polarity

The differential distribution of membrane proteins between the plasmalemmal surfaces of polarized epithelial cells enables these cells to both respond to and effect changes upon their environment in a directed fashion. The gastric parietal cell of the stomach, for example, contains a population of H,K-ATPase-rich vesicles. Upon stimulation, these vesicles fuse selectively with the luminal membrane, resulting in the massive apical secretion of HCl which initiates digestion. Without two important elements of the polarized phenotype, that is, junctional integrity and the precision of this membrane insertion, proton pumps might be delivered to a compartment which would be adversely affected by the secretion of acid. Another illustration of the utility of the polarized phenotype is provided by the principal cells of the kidney, which carry out net sodium absorption through a mechanism which is entirely dependent upon the polarized distribution of two membrane proteins. Sodium absorption is stimulated by the hormone aldosterone, which increases the amount or activity of Na,K-ATPase at the basolateral surface, while increasing the number or activity of apical sodium channels and thus the sodium conductance of the luminal membrane (Doucet and Barlet-Bas, 1989). Because the Na,K-ATPase generates low intracellular  $\{Na^+\}$ , sodium is



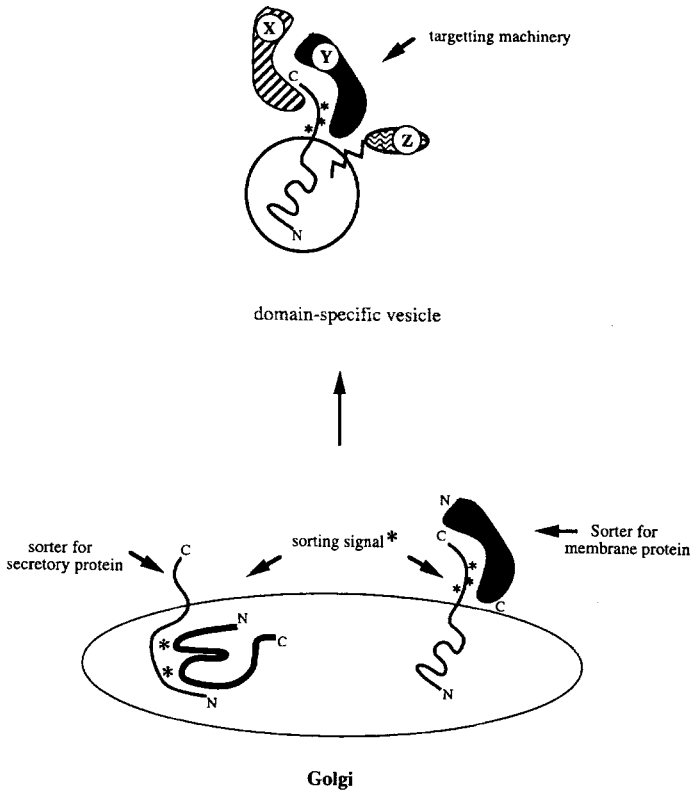
**Figure 1.** (A) Light micrograph showing the salient features of a polarized epithelium. Individual polarized epithelial cells bounded by a junctional complex (jc) come together to form a simple columnar sheet. This sheet, or epithelium, sits on a basement membrane (bm) and serves as a semi-permeable barrier between the lumen (L-continuous with the outside world of an organism) and the interstitium (In- interior) of an organism's tissues. (Photo courtesy of Dr. Marian Neutra, Children's Hospital, Boston, MA). (B) Electronmicrograph showing the unique morphological features of a polarized epithelial cell. The cell's apical (Ap) membrane surface is equipped with numerous microvillar (mv) bundles. The basolateral (Bl) domain of this particular cell is characterized by extensive interdigitations with the adjacent cell's lateral membrane. These morphologically distinct apical and basolateral membrane domains are separated by a unique ultrastructure known as the tight junction (tj). This structure is just visible as an area of close, uniform membrane apposition located at the apices between adjacent epithelial cells. (Photo courtesy of Dr. Marian Neutra, Children's Hospital, Boston, MA).

able to pass from the lumen of the kidney tubule through apical sodium channels and into the cytoplasm down its electrochemical gradient. The  $\text{Na}^+$  is then pumped across the basolateral membrane and into the interstitium by the sodium pump and is ultimately prevented from leaking back into the lumen by impermeable tight junctions. Therefore, it is the differential assignment of  $\text{Na}^+$  channels to the apical surface and  $\text{Na},\text{K}-\text{ATPase}$  molecules to the basolateral domain that ensures the vectoriality of this transport process. How the polarized cell assigns these two proteins (and apical and basolateral membrane proteins in general) to their respective surface domains has been the subject of much investigation and is the general focus of this review.

It is perhaps important to point out that the fundamental questions of plasma membrane protein anisotropy are not unique to surface membrane proteins or even to the study of epithelial polarity. The Golgi apparatus, for example, is a polarized

organelle whose *cis*- and *trans*-most cisternae are structurally and biochemically distinct. This organization is thought to enable the ordered addition and trimming of glycoprotein sugar residues as they traverse the stacked cisternae. As is clearly represented in the breadth of topics covered in this book, numerous cell types adopt a polarized state for some functional purpose. The propagation of a nervous impulse from dendrite to axon requires compositionally different membrane proteins in each of these domains, while the localization of determinants to specific parts of an egg's cytoplasm gives rise to cells with different growth potentials and the necessary asymmetries required for embryo development. What we hope will become clear in this chapter and related chapters in this book is that we are beginning to appreciate the universality of polarity. The mechanisms involved in establishing and maintaining the polarized state appear to be so fundamental that some of the schemes through which a cell is able to localize a particular protein to a given cellular domain are turning out to be conserved between epithelia and neurons, and even between epithelia and yeast.

While the need for protein asymmetries in development, or membrane polarity in epithelial transport is clear, the means through which it is achieved are only beginning to be elucidated. Before we embark upon our review of the field, we first introduce the conceptual framework onto which the results in this field are organized and interpreted. First, a protein destined to accumulate with a polarized distribution needs to be recognized as different from other proteins. We presume that what is recognized is some structural aspect of the protein itself. We refer to that part of the protein that is recognized for polarized localization as a *sorting signal* or *localization determinant*. These two terms are often used interchangeably, but in fact there is a subtle difference between the two. "Sorting signal" is often taken to imply a signal that is recognized and acted upon before the protein is delivered to its ultimate residence. Sorting signals are thought to be those signals that enable a cohort of similar proteins with similar destinations to be sorted and sifted away from all of the other molecules traversing the biosynthetic pathway at the same time. A "localization determinant" is perhaps a more general term that carries fewer mechanistic implications. It is defined here as the determinant that specifies a protein's polarized distribution, but it does not make a distinction between recognition that takes place before the protein has reached its final destination or after (e.g., through a selective retention mechanism). The proteins which serve to recognize a particular signal and act upon it are generally referred to as *sorting machinery*. Often, a distinction is made in the literature between "sorting" and "targeting machinery." In these cases, the sorting machinery is exclusively those elements which recognize the sorting signal. Any downstream effectors of this sorter that orchestrate the vectorial directing of a vesicle to its final destination are referred to as targeting machinery. A simple schematic of these elements is presented in Figure 2. As is discussed in the second half of this review, we know much more about general targeting machinery than the sorters themselves.



**Figure 2.** Conceptual framework for sorting in polarized cells. This illustration offers one of many possible ways to think about how a secretory or membrane protein could be sorted into a vesicle. It is presumed that the "sorters" will recognize a sorting signal (\*) embedded within the protein structure. It seems likely that this recognition event would need to take place in the lumen of the Golgi for a secretory protein, but this might not be necessary for a membrane protein, which could interact with a sorter from either a luminal- or cytoplasmic-facing signal domain. Ultimately, the sorted protein(s) could be contained within a "domain-specific vesicle," which would then be targetted (with the help of protein targetting machinery X, Y, and Z) to the appropriate apical or basolateral surface domain.

## B. Sorting Pathways in Polarized Cells

It is thought that proteins destined for either the apical or basolateral domain of a polarized cell occupy the same Golgi cisternae during their biosynthesis (Matlin and Simons, 1984; Misek et al., 1984; Rindler et al., 1984; Fuller et al., 1985; Pfeffer et al., 1985). Immunoelectron microscopic studies performed on nonpolarized

endocrine cells which manifest two biochemically and kinetically different secretory pathways suggested that the process of sorting components away from one another takes place at the TGN (Orci et al., 1987; Tooze et al., 1987). However, recent studies have demonstrated that sorting may not take place exclusively at the TGN. Sorting mechanisms have been suggested to take effect as early along the biosynthetic pathway as the ER (Balch et al., 1994) as well as at the recycling endosome (Matter et al., 1993; Matter and Mellman, 1994). In hepatocytes, sorting appears to occur after all newly synthesized membrane proteins are delivered to the basolateral plasmamembrane (Bartles et al., 1987). Similar delivery routes have been detected in polarized intestinal epithelial cell lines (Matter et al., 1990). Finally, in at least one subclone of the canine renal MDCK cell line, sorting may take place both at the Golgi as well as at the level of the plasma membrane. While most proteins in this cell line are sorted in the TGN, the Na,K-ATPase can be preferentially localized to the basolateral membrane through domain-specific stabilization mechanisms *after* random insertion into both plasmamembrane domains (Hammermerton et al., 1991; Siemers et al., 1993).

Apically and basolaterally sorted proteins have been shown to be packaged into distinct classes of Golgi-derived vesicles (Wandinger-Ness et al., 1990) which are ultimately targeted to their appropriate domains. Recently it has been shown that membrane and secretory proteins are segregated into distinct vesicular carriers upon transport from the Golgi to the basolateral surface of hepatocytes (Saucan and Palade, 1994) The extent to which distinct basolateral (or apical) proteins are co-sorted and incorporated within the same vesicle either due to common localization signals or the ability to co-aggregate has not yet been determined.

After proteins are sorted, the targeting of a vesicle to a particular surface domain can occur directly (vectorially) from the TGN to the apical domain (Matlin and Simons, 1984; Rindler et al., 1984; Fuller et al., 1985), basolateral domain (Caplan et al., 1986) or indirectly as has been shown for the poly-immunoglobulin receptor (pIgR) (Mostov and Deitcher, 1986). In the latter case, the protein is first targeted to the basolateral surface where the receptor can bind its ligand and is then transported to the apical surface via a process known as transcytosis (reviewed in Mostov and Simister, 1985). As noted above, in hepatocytes all apical proteins studied to date make use of this indirect pathway for apical delivery (Bartles et al., 1987), while cell lines derived from intestine and kidney can employ both routes for surface delivery (Matter et al., 1990; Casanova et al., 1991; Low et al., 1991) While the details of the routes have been determined for a number of sorting pathways, the molecular signals and recognition components which control each of them are not well understood.

The search for these molecular signals and recognition components has been the focus of much study over the last 15 years. During this period, the subjects of protein sorting and epithelial polarity have been extensively reviewed. Several of these reviews are listed here for those seeking more background on specific aspects of this field: for general reviews on protein sorting pathways (Burgess and Kelly,

1987); general concepts of sorting and targeting (Caplan and Matlin, 1989); a discussion of the mechanisms required for the establishment and maintenance of epithelial polarity (Rodriguez-Boulau and Nelson, 1989); polarized transport of surface proteins and lipids in epithelial cells (Simons and Wandinger-Ness, 1990); comparative epithelial and neuronal polarity (Rodriguez-Boulau and Powell, 1992); the generality of the polarized phenotype (Nelson, 1992); cytoskeleton as a component of the protein sorting machinery (Mays et al., 1994); summary of the few known sorting signals in polarized epithelial cells (Mostov et al., 1992); common signals involved in sorting from the TGN and endosomes (Matter and Mellman, 1994).

Perhaps now more than ever before, it is becoming a rather daunting task to provide a synthesis of the observations relevant to the study of epithelial polarity. This is in part due to the fact that important insights into the mechanisms of sorting are being contributed by fields that are not exclusively focussed on epithelial biology. As we discussed in this review, some important contributions are emerging from studies of endocytosis, secretion in yeast and neurons, and the sorting of yeast lysosomal enzymes (see Chapter I of this volume), in addition to more "classical" approaches to epithelial polarity. In this review, we explore the current paradigm that the generation and maintenance of distinct membraneous compartments requires "sorting signals," the recognition domains embedded within the amino acid sequence or polypeptide structure of the protein, and "sorting machinery," the proteins which interpret and act upon these signals. In the first half, we review and categorize the signals that have begun to be elucidated, as well as discuss the approaches and difficulties associated with finding and interpreting sorting signals. While the polarity field itself has not yet succeeded in characterizing the definitive sorting machinery, numerous components of the membrane budding and fusion apparatus are rapidly being elucidated. We have chosen to review some of the important findings in the field of membrane transport, and in particular examine the potential roles that GTP-binding proteins of the rab, ARF and heterotrimeric classes may play. We also discuss a class of proteins referred to as adaptins as well as the implications that the SNARE hypothesis may have for epithelial polarity. Although these components have not been shown to be directly involved in sorting per se, it is becoming increasingly clear that in a general sense, the composition of the membrane vesicle budding and fusion machinery may be part of the overall apparatus which "acts upon" the sorted species and contributes to domain specific surface targeting.

### C. The Sorting Signal Paradigm

As stated above, the paradigm for conceptualizing the mechanisms responsible for biosynthetic sorting requires that each protein contains signal information embedded within its polypeptide sequence/structure (sorting signal) which is interpreted and acted upon by components referred to as sorting machinery. This scheme



takes its cue from the process through which ribosomes translating secretory and membrane proteins are targeted to the endoplasmic reticulum to initiate co-translational protein translocation (Blobel, 1980). Prior to the elucidation of this process, it was suggested that protein targeting might require cellular sorting machinery to recognize certain signals which would be shared by proteins with common destinations (Blobel, 1980). Shortly after this suggestion, it became clear that targeting to the RER, mitochondria and chloroplasts required short, contiguous, *N*-terminal signal peptides (reviewed in Burgess and Kelly, 1987). In the case of the former, the signal was recognized by a receptor, SRP (Lingappa et al., 1978; von Heijne, 1984; Kurzchalia et al., 1986; Walter and Lingappa, 1986). Subsequently, a number of short, contiguous amino acid domains have been shown to play a role in later stages of post-synthetic targeting. These include: (1) the KDEL and adenovirus E19 signals which ensure the retention or recapture of resident ER proteins (Munro and Pelham, 1987; Nilsson et al., 1989); (2) a transmembrane domain signal responsible for Golgi retention (Swift & Machamer, 1991; Machamer, 1993); (3) the cluster of positively charged lysine residues (SV40-NLS) sufficient for nuclear targeting (Richardson et al., 1986); (4) the critical tyrosine/"tight-turn" structural motif which can mediate localization to clathrin coated-pits (Goldstein et al., 1985; Pearse and Robinson, 1990; Collawn et al., 1991); and (5) the discovery that lysosomal hydrolases were targeted to lysosomes through the recognition of a phosphorylated sugar residue (mannose-6-phosphate; reviewed by Kornfeld and Mellman, 1989). In several of these cases receptors for these signals have been well-characterized: the signal recognition particle (SRP) for secretory and membrane proteins (Walter and Lingappa, 1986), the mannose-6-phosphate receptor (M6PR) for the targeting of lysosomal hydrolases to the lysosome (Sly and Fischer, 1982; von Figura and Hasilik, 1986), the KDEL receptor (Tang et al., 1993) and the adaptins which couple coated pit localization sequences to clathrin cages (Pearse and Robinson, 1990; Robinson, 1994).

## II. SORTING SIGNALS IN EPITHELIAL MEMBRANE POLARITY

### A. Considerations Relevant to the Study of Sorting Signals

The search for definitive signals which mediate the delivery of proteins to a particular epithelial surface domain has proven to be quite difficult. This is due in part to general limitations imposed by certain molecular biological approaches, as well as to some inherent difficulties specific to the investigation of epithelial polarity. Our goal in this section is to outline reasonable criteria for the identification of a sorting signal.

The observation that the influenza and vesicular stomatitis viruses bud from opposite surface domains of polarized MDCK cells (Madin Darby Canine Kidney)

(Rodriguez-Boulan and Sabatini, 1978) spawned an extensive search in which chimeric and deletion analyses were applied to the problem of identifying the underlying apical and basolateral sorting signals (reviewed in Caplan and Matlin, 1989). These efforts to characterize sorting signals have generally involved the generation of chimeric or truncated constructs prepared from portions of apical and basolateral membrane proteins. Through analysis of the subcellular distributions of the resulting proteins, sorting information can, at least in theory, be assigned to particular portions of the parent molecules. While a large number of chimeric and truncated viral glycoproteins have been generated and analyzed, it has been difficult to interpret many of the resultant observations. With the benefit of hindsight, we now know that these difficulties can be attributed to a number of issues that we discuss in more detail below (including the tertiary structures of the experimental constructs, the confounding possibilities introduced by uncharacterized default pathways, and the potential for multiple and hierarchical signals to be embodied within the structures of the studied proteins). Until recently (Thomas and Roth, 1994), the analysis of viral spike glycoproteins did not produce a definitive sorting signal. Much of the uncertainty associated with this work is likely attributable to the fact that these studies engineered chimeras from portions of structurally dissimilar molecules. The tertiary structures of the resultant chimeras may thus differ substantially from those of either parent molecule, which may in turn exert unpredictable effects upon sorting behavior. Clearly, if sorting signals are formed from domains arising from noncontiguous regions of a polypeptide, for example, in much the same manner that heterotrimeric G proteins are thought to "see" their effectors (Berlot and Bourne, 1992), or in the way that the human growth hormone receptor (hGHbp) is thought to interact with its ligand (Cunningham and Wells, 1989), it is easy to imagine how the structural integrity of the putative sorting signal could become compromised in a chimeric construct.

While producing a rough map of the signal-bearing domain of a protein can be relatively straight forward, determining the exact residues which constitute the signal is turning out to require a collaboration between many different types of mutagenesis approaches. Often, contradicting results can arise from alanine scanning, truncation and point mutation/deletion mutagenesis, since a mutated protein can manifest impaired sorting behavior even though the altered residues are not part of the actual sorting signal (Aroeti et al., 1993). It is becoming clear that a judicious and thorough comparison of many different types of mutagenesis approaches may be necessary to determine definitively the key residues necessary for sorting.

#### B. "Default" Sorting Pathways and the Interpretation of Sorting Signals

Perhaps another difficulty in looking for apical or basolateral sorting signals is that the default pathway for "signal-less" membrane proteins is still not known. A protein that is sorted "by default" is, by definition, unable to interact with and be

acted upon by any sorting machinery whatsoever. In theory, at least, such "unsorted" proteins may be distributed with polarity, depending on the nature and characteristics of the membrane vesicular traffic arising from the Golgi complex in a particular cell type. Obviously, if the localization of a protein construct under study is identical to that produced by the cell's default pathway, elucidation of a signal will be difficult, since elimination of the signal will not alter the protein's distribution. Thus, one can appreciate the difficulty in assigning localization information to a particular domain in the context of an undefined default pathway. This caveat accounts for at least some of the reasons which explain why a definitive basolateral sorting signal in the C-terminal domain of VSV-G protein took so long to discern. In the following example we summarize the HA-VSVG spike glycoprotein chimera literature as a means to illustrate the difficulties in interpreting these types of studies.

When a cDNA encoding the influenza HA was expressed in MDCK cells, the encoded protein localized to the apical membrane (Roth et al., 1983), while a cDNA encoding the VSVG polypeptide produced a protein that is localized to the basolateral domain (Gottlieb et al., 1986b; Stephens and Compans, 1986). When truncation mutants were expressed in which soluble ectodomain versions of these proteins were synthesized, the VSVG ectodomain was secreted from both apical and basolateral domains (Stephens and Compans, 1986; Gonzalez et al., 1987) while the HA ectodomain was predominantly secreted from the apical domain (Gonzalez, et al., 1987; Roth et al., 1987b). Based on evidence that the default pathway for secreted proteins leads to nonpolarized secretion from both surface domains (Kondor-Koch et al., 1985; Gottlieb et al., 1986a; Caplan et al., 1987), it was reasoned that the ectodomain of HA encodes an apical sorting signal while the VSVG ectodomain lacks signal information. This was further confirmed by the observation that a hybrid HA-VSVG protein comprising the HA ectodomain fused to the VSVG transmembrane and cytoplasmic tail region was targeted to the apical membrane (McQueen et al., 1986; Roth et al., 1987a). But if the VSVG ectodomain is randomly secreted and the VSVG tail domain fused to HA is apical, which domain of VSVG encodes basolateral sorting information? The complementary hybrid comprised of the ectodomain of VSVG (presumably signal-less) tethered to the HA transmembrane and tail region (perhaps also signal-less) was targeted either to the basolateral membrane or to both surface domains (McQueen et al., 1986; Puddington et al., 1987; Roth et al., 1987a; Compton et al., 1989). The interpretation of the behavior of this chimera was clearly complicated; it was suggested that this protein could be pursuing its distribution by default. (As discussed above, the default pathway for membrane proteins is still not defined in polarized cells).

An alternative interpretation was that the VSVG ectodomain indeed contains basolateral sorting information, but that perhaps this domain needs to be tethered to the plasmamembrane with a transmembrane anchor in order to interact with its presumptive sorting machinery. This interpretation, however, was proved incorrect by the observation that the anchoring of this ectodomain to the membrane through a

lipid-linkage resulted in apical targeting (Brown et al., 1989). Interestingly, when the ectodomain of the normally apical placental alkaline phosphatase (PLAP) was attached to the VSVG transmembrane and cytosolic tail domains (which were thought to lack a dominant signal), the resulting chimeric protein was targeted basolaterally. It is difficult to reconcile the HA-VSVG and PLAP-VSVG chimeras without invoking hierarchical and competing signals. Recently, a basolateral targeting signal has been precisely localized to the cytoplasmic domain of the VSVG protein (Thomas and Roth, 1994). In light of the vicissitudes which attended the interpretation of each round of chimeric constructs discussed above, it was certainly unexpected that definitive sorting information would be localized to the cytoplasmic tail of VSVG. The nature and function of this signal will be discussed in depth below. The preceding discussion was presented simply to reinforce the caveat that the default pathway, protein structural considerations and the possible interactions between "dominant" and "recessive" sorting signals can considerably cloud the interpretation of chimera experiments.

### C. Multiplicity of Signals and Epithelial Polarity

Recent studies of the polymeric immunoglobulin receptor (pIgR), the low density lipoprotein receptor (LDLR) and polytopic hetero-oligomeric proteins (H,K-ATPase and Na,K-ATPase) suggest that individual proteins can interact in multiple and complex fashions with the machinery responsible for surface targeting. It is becoming increasingly clear that there can be an array of signals encoded within an individual protein, and the sorting problem is becoming evermore complicated by the apparent redundancy, multiplicity and hierarchical nature of these signals (Matter et al., 1992; Mostov et al., 1992). For example, Brewer and Roth's (1991) demonstration that they could completely overwhelm the apical signal present in the HA ectodomain and redirect it to the basolateral surface by changing a single amino acid in this protein's cytoplasmic tail strongly suggests that multiple signals present in a single protein can interact in a hierarchical fashion. The newly created cytoplasmic signal is dominant over the presumed apical sorting signal present in the ectodomain of HA.

As discussed below, the LDL receptor has been shown to encode redundant, basolateral sorting information, since either of two cytoplasmic determinants could independently mediate basolateral delivery (Matter et al., 1992). Moreover, the protein may also contain a cryptic apical sorting signal in its ectodomain, since a cytoplasmic tail-minus construct of this protein (CT12) is sorted with great efficiency to the apical membrane in MDCK cells (Matter et al., 1992). An ectodomain apical localization signal has also been found within the pIgR, whose initial surface delivery is to the basolateral plasmalemma. Why do these proteins need multiple signals? What does the LDLR gain by expressing two basolateral localization signals? Recent studies (discussed in greater detail in the following section) have more finely decoded these two signals and are revealing functional differences. For ex-

ample, the "membrane proximal determinant" encodes coated-pit internalization information, while the "membrane distal determinant" appears to ensure efficient sorting from a basolateral endosome back to the basolateral surface (Matter et al., 1993).

Analysis of the sorting behavior of multisubunit ion pumps provides further insight into the possible utility of multiple signals (reviewed in Gottardi et al., 1993). The gastric H,K-ATPase and the Na,K-ATPase are close cousins in the large family of P-type ion transporting ATPases. Both are composed of 100 kDa  $\alpha$ -subunits and heavily glycosylated 55 kDa  $\beta$ -subunits. They share similar reaction mechanisms and catalytic properties and, not surprisingly, are highly homologous at the amino acid sequence level. The  $\alpha$ -subunits are ~65% identical, whereas the  $\beta$ -polypeptides manifest roughly 40% identity. While the Na,K-ATPase is a basolateral protein in most polarized epithelial cell types (with the exception of neural epithelia such as choroid plexus and retinal pigment epithelium), the H,K-ATPase occupies the apical membrane and a pre-apical storage compartment in gastric parietal cells. Hormonal stimulation of gastric acid secretion induces fusion of the membrane vesicles which comprise the intracellular reservoir with the plasma membrane, resulting in delivery of the H,K-ATPase to the apical cell surface. During the interdigestive period, the H,K-ATPase is re-endocytosed and returned to its storage compartment. Chimera studies reveal that each subunit of the H,K-ATPase possesses a sorting signal which participates in regulating this complex traffic (Gottardi and Caplan, 1993). The  $\alpha$ -subunit is endowed with a dominant apical targeting signal, which can drive the apical sorting of chimeric pumps expressed in both MDCK and LLC-PK1 renal epithelial cells. The  $\beta$ -subunit of the H,K-ATPase possesses a tyrosine-based endocytosis signal (Roush et al., manuscript submitted). This signal causes the protein to be sorted basolaterally when it is expressed in MDCK cells and apically when it is expressed in LLC-PK1 cells. The Na,K-ATPase  $\beta$ -subunit does not possess a similar sequence domain. It seems likely that the two H,K-ATPase signals participate in distinct stages of pump sorting in the gastric parietal cells. The apical signal in the  $\alpha$ -subunit probably mediates the sorting of the entire complex to the apical membrane or the pre-apical storage compartment, whereas the  $\beta$ -subunit signal is responsible for ensuring the re-internalization of the pump following the cessation of secretagogue stimulation (Courtois-Coutry et al., 1997). It remains to be determined why the  $\beta$ -subunit's tyrosine-based signal is differentially interpreted by MDCK and LLC-PK1 cells. Investigation of this phenomenon may shed light on the nature and function of the epithelial sorting machinery.

This apparent trend towards a multiplicity of signals is not entirely surprising, since many proteins are required to perform highly sophisticated feats of membrane targeting during the course of their transits throughout the endomembranous networks of the cell. For example, the pIgR receptor expressed in its native hepatocytes or by transfection in MDCK cells travels first to the basolateral membrane to pick up ligand and is then transported to the apical surface domain. It appears that an apical sorting signal in this protein's ectodomain might be required for basolateral to

apical transcytosis, while a basolateral signal in the cytoplasmic domain ensures the initial basolateral delivery. Unlike proteins that are constitutively expressed at one surface domain, a number of distinct and individually acting signals are necessary to orchestrate the more complicated surface targeting events displayed by pIgR receptor, and other molecules like it. Obviously, the hierarchical (both temporal and spatial) regulation of each signal will be of utmost importance in ensuring that a protein follows a physiologically relevant trafficking pathway. Recent evidence, for example, demonstrates that the pIg receptor undergoes phosphorylation on a cytosolic serine residue around the time that it is delivered to the basolateral surface (Larkin et al., 1986). This phosphorylation event appears to inactivate the protein's basolateral signal and thus permit its transcytosis to the apical membrane (Casanova et al., 1990).

#### D. The Discovery of Distinct Basolateral Targeting Determinants

Perhaps not surprisingly, the greatest advances in the elucidation of sorting signals have been made with single membrane-spanning monomeric or homooligomeric proteins (e.g., pIgR, LDL-R, TfR). With these molecules the requirements for surface expression are easily met and the effects of mutagenesis on tertiary structure can be assessed through well-characterized functional assays, such as receptor-ligand or antibody binding. Through deletion analysis and heterologous expression in MDCK cells, it was determined that the pIgR (Casanova et al., 1991) and the LDLR (Hunziker et al., 1991) each contained basolateral targeting determinants which mapped to short, contiguous regions of their cytoplasmic domains (Table 1). Both signals could be grafted onto heterologous proteins and cause them to be targeted to the basolateral surface, supporting the idea that each determinant was truly an autonomous basolateral sorting signal. Exhaustive mutagenesis studies have more finely mapped each of these determinants. The LDLR possesses two distinct basolateral targeting determinants, one that is "coated-pit related" (proximal determinant) and another which is tyrosine-dependent but not capable of mediating localization into coated-pits (distal determinant) (Matter et al., 1992; 1993; 1994). Interestingly, the polymeric immunoglobulin receptor (pIgR) signal may constitute yet another class of basolateral targeting determinant, since it shares little in the way of sequence homology with either determinant of the LDLR and shows weak tyrosine dependence (Aroeti et al., 1993). The general characteristics of these three determinants and the degree to which they are related are only beginning to be elucidated (Matter et al., 1994; Thomas and Roth, 1994). An attempt to categorize these basolateral sorting determinants has been made by Matter et al. (1994) and is summarized in Table 1.

Before discussing the nature of the "coated-pit related" basolateral targeting determinant, it is necessary to be familiar with the signals that are known to mediate the accumulation of plasma membrane receptors into clathrin-coated pits (Goldstein et al., 1985). It is now generally accepted that tyrosine- and dileucine-

**Table 1.** Classification of Basolateral Sorting Determinants**Coated Pit Unrelated Localization Signals***Strong Tyrosine Dependence*

LDL receptor- distal determinant                      LDL-R (distal)            -- Q D **G** Y S Y P S R Q M V S L **E** **D** **D** V A --  
 Transferrin receptor (TfR)

*Weak Tyrosine Dependence*

pIg receptor (pIgR)                                      pIgR            -- **R** **H** **R** **R** **N** **V** **D** **R** **V** **S** **I** **G** **S** **Y** **R** T -- including downstream acidic residues

**Coated-Pit Related Localization Signals***Tyrosine Dependent*

LDL receptor- proximal determinant                      LDL-R (proximal)            -- N F D N P V **Y** Q K T T **E** **D** **E** V H --  
 Hemagglutinin (HA)-Y543                              HA-Y543            -- N G S L Q **Y** R I C I --  
 VSV G    VSV G            -- C I K L K H T K K **R** Q I Y T D I E M --  
 Lysosomal membrane glycoproteins (Igp-A/lamp-1)                      Igp-120            -- K R S H A **Q** Y Q T I --  
 Lysosomal acid phosphatase (LAP)                      LAP            -- Q A Q P P G **Y** **R** H V A **D** G E D H A --  
 Asialoglycoprotein receptor (ASGP-R)                      ASGP-R (H1 subunit)            M T K E Y Q D L Q H L D N E E S D H H --  
 Nerve growth factor receptor (NGF-R)

*Tyrosine Independent*

IgG Fc receptor (di-leucine dependent)                      FcRII-B2            -- **N** **I** **I** **T** **Y** **S** **L** **L** **K** **H** -- (including downstream acidic residues)

**Notes:** This table was adapted from that of Matter et al., 1994 and Matter and Mellman, 1994. The classification of signals (e.g., coated-pit related, unrelated, etc.) is shown on the left column, while the actual signal for most of the proteins is shown on the right. Critical residues for basolateral sorting are shown in bold face type, while other important residues are underlined. The dashes (-) present over the residues E D D of the LDL-R (distal) signal and the E D E of the LDL-R (proximal) signal denote that these amino acids are acidic and important for sorting signal function.

containing sequence motifs present in the cytoplasmic tails of a number of coated-pit clustering proteins serve as the critical recognition elements for the adaptor components of clathrin coats (Pearse and Robinson, 1990; Trowbridge, 1991). More recently, numerous studies have demonstrated a strong relationship between the signals which mediate localization into coated pits and a subset of those involved in basolateral targeting (Brewer and Roth, 1991; Hunziker et al., 1991; LeBivic et al., 1991). For example, Brewer and Roth (1991) found that the apically targeted HA molecule could be completely rerouted to the basolateral membrane by replacing a strategically localized cysteine residue (cys 543) with a tyrosine in the cytoplasmic domain. This tyrosine was also sufficient to localize this protein into coated-pits and direct the protein's incorporation into endosomes. This observation that an endocytosis signal might also double as a basolateral targeting signal led to the suggestion that the recognition determinants for endocytosis and for TGN-to-basolateral targeting might be similar or identical to one another. Thorough mutagenesis studies on the coated-pit localization and basolateral sorting determinants of HA-Y543 (Thomas and Roth, 1994; Lin et al., 1997), VSVG protein (Thomas et al., 1993), and the LDLR (Matter et al., 1994), however, have led to a revision of this initial interpretation. It turns out that the "endocytosis signal" of both the HA-Y543 and the LDLR (proximal signal) can be resolved into two overlapping but distinct signal components. In other words, there is information recognized for endocytosis that is distinct from that recognized for basolateral sorting, even though the sequences are in part superimposed and share marked similarity. Table 2 shows the systematic mutagenesis that ultimately unraveled this relationship. Brewer and Roth (1991) found that HA-Y543 is capable of both basolateral sorting and endocytosis. The second generation mutant HA-Y543,R546, however, behaved as a protein that was capable of endocytosis, but whose basolateral localization was inhibited (Lin et al., 1997). Similar results were found with the LDLR proximal determinant. Matter and colleagues (1994) showed that the truncation mutant CT27 was basolaterally targeted and rapidly endocytosed, while the removal of terminal acidic residues in CT22 pro-

**Table 2.** Evidence that Coated-Pit-Related Determinants Encode Two Distinct Signals.

<i>Protein</i>	<i>Signal Region</i>	<i>Cell Surface Localization</i>	<i>Endocytosis Competent?</i>
HA	—N G S L Q C R I C I	apical	no
HA-Y543	—N G S L Q Y R I C I	basolateral	yes
HA-Y543, R546	—N G S L Q Y R I R I	inhibited	yes
LDL-R (Proximal Determinant)			
CT-27	—N F D N P V Y Q K T T E D E V H	basolateral	yes
CT-22	—N F D N P V Y Q K T T	apical	yes

**Notes:** This table summarizes data from Thomas and Roth, (1994) and Matter et al., (1993) demonstrating that the coated-pit related basolateral sorting determinant could be resolved into two distinct signals, one which mediates basolateral localization, the other which mediates endocytosis.



duced a protein that was not capable of basolateral targeting, but could nonetheless be endocytosed. Thus, the initial correlation between endocytosis signals and basolateral targeting has now resolved into two distinct but overlapping signals that can share common residues for their respective activities.

The implications of this result are very exciting for the field of epithelial polarity. First, they suggest that the signals for basolateral sorting/targetting may be structurally similar to signals for clathrin-coated pit localization and endocytosis. The involvement of similar signals suggests that the sorting/recognition molecules themselves may be related. At least for endocytosis signals, there is evidence in favor of clathrin "adaptins" (of the AP2 plasma membrane class) playing a role in recognizing these sequences (Pearse, 1988; Glickman et al., 1989; Beltzer and Spiess, 1991; Sorkin and Carpenter, 1993; Sosa et al., 1993). In light of the recent characterization of adaptin related molecules (COPs, discussed in section III, below), it has been suggested that a family of structurally and functionally similar sorting adaptors may serve as the sorting machinery which interacts with these basolateral sorting signals (Matter et al., 1994). The findings support the more general contention that sorting at the level of the TGN may be mechanistically similar to that at the level of the endosome (Matter et al., 1993, 1994).

Taken altogether, there now appear to be two general classes of basolateral targeting determinants. One of these is biochemically related to the signals that mediate sorting into coated pits. This type of signal can be colinear with an endocytosis determinant and may share the critical tyrosine residue required for the activity of both, but it is nonetheless distinct and dissociable from an endocytosis signal. The second class of basolateral targeting determinants appears to be unrelated to clathrin-coated pit localization signals, although it may also strongly depend on a tyrosine for activity. This second type of determinant appears to be unique to the LDLR, pIgR (Casanova et al., 1991) and the TTR (Dargement et al., 1993), although these signals share no primary sequence homology with one another. It is possible however, that this second determinant present in these three proteins may be mutually similar in three-dimensional structure but not in primary sequence. In this context it is important to note that adaptor proteins are thought to recognize tyrosine residues in the context of a tight turn, which can be achieved by many different primary sequences (Glickman et al., 1989; Collawn et al., 1990, 1991; Bansal and Gierasch, 1991). More detailed analyses are revealing that while the dependency on tyrosine is crucial, other residues which are acidic and C-terminal to the tyrosine are also important. Matter et al. (1994) demonstrated that the clusters of two or more acidic amino acids downstream from a tyrosine, phenylalanine or di-leucine are important for signal function (see Table 1). While the authors of this study have argued that it is premature to propose a common motif characteristic of all basolateral targeting determinants, they have found that this critical aromatic amino acid followed by acidic residues can be discerned in the cytoplasmic domains of many known proteins which are targeted to the basolateral membrane of MDCK cells, including E-cadherin, transferrin receptor, cation-independent and dependent mannose-6-

phosphate receptors, LAP, pIgR and FcRIIB2. (see discussion of Matter et al., 1994). As these authors have suggested, it will be exciting to define mutations that will prevent the recognition of these sequences so that the identification and characterization of the molecules which serve to interact with and interpret these signals can be facilitated.

### E. Apical Sorting: GPI-linkages and Glycosphingolipids

An ever growing list of proteins are anchored to membranes through a covalent attachment to glycosylphosphatidylinositol or GPI. Proteins of this class are initially synthesized on bound polysomes as transmembrane polypeptides and, while still resident within the ER, are cleaved from their transmembrane portions and transferred covalently to lumenally facing glycosyl-phosphatidylinositol molecules (Cross, 1990). GPI-anchored proteins are widely distributed with respect to both cell type and function. Members of this class of proteins include protozoal surface coat proteins (e.g. the variant surface glycoproteins of trypanosomes), differentiation antigens (e.g., Thy-1), adhesion molecules (e.g., the GPI-linked isoform of N-CAM), hydrolases (e.g., alkaline phosphatase and 5'nucleotidase), and receptors (folate receptor). The functional advantages that this membrane linkage confers upon a particular protein is presently unclear, and has been the focus of a great deal of attention (reviewed in Brown, 1992). In general, the GPI-linkage has been suggested to be important for enabling proteins to "cluster" at a surface density much higher than is possible for single-pass transmembrane proteins (Hooper, 1992). Studies have also shown that these clusters of GPI-anchored proteins may be important for certain cell surface signal transducing events (reviewed in Anderson, 1993).

GPI-linked proteins captured the attention of epithelial biologists because of their polarized distribution in MDCK cells (Lisanti et al., 1988) and other cultured epithelial cell lines (Lisanti et al., 1990). The nearly exclusive correlation of membrane anchoring via GPI with apical localization raised the question as to whether or not the GPI membrane anchor was itself a signal for apical targeting. Chimeric analyses showed clearly that the GPI-linkage is sufficient for apical targeting in MDCK cells (Brown et al., 1989; Lisanti et al., 1989a,b). Of course, in the absence of a known default pathway for membrane proteins, it remains formally possible that the GPI-anchor prevents a protein from gaining entry into the basolateral sorting pathway. Moreover, the fact that the cytoplasmic tail-minus versions of the LDL and pIg receptors are directly targeted to the apical membrane is consistent with the possibility that apical sorting occurs by default (discussed in Matter and Mellman, 1994). Nonetheless, the GPI-linkage is the field's best accepted apical localization signal characterized to date. Interestingly, glycosphingolipids (GSLs) share the apical preference of GPI-linked proteins and are generally found exclusively in the outer leaflets of the apical membranes of MDCK cells.

The means through which GPI-anchored proteins and glycosphingolipids (GSLs) are sorted and subsequently targeted to the apical membrane are poorly un-

derstood. It has been shown that GSLs manifest biophysical properties which enable them to self-associate or form clusters in the plane of the membrane (Thompson and Tillack, 1985). These properties have been invoked to support the proposal that GSL clustering occurs at the level of the TGN, and that newly synthesized GPI-linked proteins might co-cluster with these lipids (Simons and van Meer, 1988). It has been further suggested that apically-destined transmembrane proteins could similarly be sorted through an ability to co-cluster with GSLs and GPI-linked proteins (Simons and Wandinger-Ness, 1990). According to this model, apical sorting could take place through selective inclusion within these GSL microdomains, while certain basolateral membrane protein components would be sorted by selective exclusion. However, it should be pointed out that there is still no experimental evidence showing that the GSL clusters are important for apical sorting. One cell line in particular suggests that the role of GSLs in sorting of GPI-anchored proteins may be more complex. A rat thyroid epithelial cell line (FRT) distributes its GSLs and GPI-anchored proteins to the basolateral surface while the polarized distribution of a number of transmembrane proteins is identical to that of MDCK cells (Zurzolo et al., 1993). This suggests that at least some of the apical proteins analyzed (e.g., HA) do not partition with basolaterally directed GSLs. The FRT cell line will serve as an excellent tool for furthering our understanding about the role of glycolipid clustering in the sorting of proteins and lipids in polarized epithelial cells.

#### F. Tissue and Cell-Type Specificity of Membrane Polarity

Most of the early studies in epithelial polarity used the kidney-derived MDCK cell line as their workbench. However, the last six years has seen the introduction of a number of new cell culture models into the field: CaCo2 (Pinto et al., 1983; Matter et al., 1990; Costa de Beauregard et al., 1995); HT-29 and T-84 (human intestinal epithelial), (Madara et al., 1987; Polak-Charcon et al., 1989; Mikogami et al., 1994); LLC-PK1 (pig kidney proximal tubule) (Hull et al., 1976; Gstrauchaler et al., 1985; Gottardi and Caplan, 1993; Gottardi et al., 1995); MDBK (Madin-Darby bovine kidney) (Furuse et al., 1994), FRT (Fischer rat thyroid) (Zurzolo et al., 1993), as well as primary cultures of choroid plexus and retinal pigmented epithelium (Marrs et al., 1993). As we have discussed in the first half of this review, we are just beginning to elucidate the nature of certain "apical" and "basolateral" sorting signals. However, the "nonstandard" sorting of GPI-link proteins in FRT cells mentioned above, and the fact that a number of proteins display tissue and cell-type specific membrane localizations (see Table 3), calls into question the ways in which we think about polarized sorting signals and the mechanisms of sorting.

As shown in Table 3, there are notable differences in the localization of certain membrane proteins expressed in different tissue cell-types. The Na,K-ATPase, nearly ubiquitously expressed at the basolateral domain of most polarized cell types, is localized to the luminal (apical) domain of both retinal pigmented epithelial and choroid plexus cells (Wright, 1972; Steinberg and Miller, 1979; Spector and

**Table 3.** Tissue Specificity of Sorting

Protein	Tissue/Cell Type	Apical	Basolateral	Axon	Dendrite	Ref.
LDL-R	intestine		+			a
	liver		+			a
	proximal kidney	+				a
Na,K-ATPase	intestine, liver, kidney		+			b
	choroid plexus	+				c
	retinal pigmented epithelium	+				d-e
	neuron			+	+	f
	most polarized cell culture models, e.g., MDCK I & II, LLC-PK1, Caco-2, native hepatocytes	+				g-i
GPI-linked proteins	Fischer Rat Thyroid (FRT) epithelial cells		+			j
	Neuron			+		k
	Follicular Epithelium of <i>Drosophila</i>		+			l
	Gut epithelium of <i>Drosophila</i>	+				l
vacuolar H <sup>+</sup> ATPase	kidney: alpha intercalateed cells	+				m-n
	beta intercalated cells					m-n
	intercalated cells		+			

**References:** (a) Pathak et al., 1990; (b) Almers and Stirling, 1984; (c) Quinton et al., 1973; (d) Steinberg and Miller, 1979; (e) Rizzolo, 1990; (f) Pietrini et al., 1992; (g) Lisanti et al., 1988; (h) Lisanti et al., 1989a,b; (i) Lisanti et al., 1990; (j) Zurzolo et al., 1993; (k) Dotti et al., 1991; (l) Shiel and Caplan, 1995a,b; (m) Schwartz et al., 1985; (n) Brown et al., 1988.

Johanson, 1989; Gundersen et al., 1991). When the cDNA encoding the LDL receptor was placed under the control of a metallothionein promoter and employed in the generation of a transgenic mouse, the receptor was expressed at the basolateral domains of liver and intestinal epithelial cells, but unexpectedly localized to the apical domains of proximal kidney tubule cells (Pathak et al., 1990). The polarized budding of certain viruses and the localization of their respective spike glycoproteins was shown to vary considerably between kidney derived MDCK and thyroid-derived FRT cells (Zurzolo et al., 1992a). In some instances, a shift in the type of targeting pathway used by a protein can depend on the differentiated state of the cell culture (Zurzolo et al., 1992b). Furthermore, the polarized localization of a particular GPI-linked protein was found to be developmentally regulated in *Drosophila* embryos (Shiel and Caplan, 1995a). Finally, a remarkable flexibility and "plastic-

ity" of protein sorting has been suggested to be present in kidney intercalated cells, which appear to direct the vacuolar proton pump to either surface domain, depending on particular environmental cues (Schwartz et al., 1985; Brown et al., 1988a)

At the present time, we have little understanding of the signals or sorting mechanisms that mediate the differential sorting of the same protein in distinct cellular types. Are different signals recognized by the different epithelial cells or is the same signal interpreted differently? Is the sorting machinery itself different between polarized cells, or is the sorting machinery basically conserved between different cell-types while its regulation, adaptation, or wiring to the targeting machinery is different? Evidence discussed in the second half of this review on the rab family of proteins suggests that elements of the targeting machinery are in fact highly conserved between different cell types, and it is the cell-type specific adaptation of this machinery which accounts for differences. Nonetheless, it is becoming clear that the sorting of a particular protein can be a highly idiosyncratic feature of each polarized cell model.

The observation that different epithelial cell lines may handle the same protein (or the same signal) differently has to reflect more than a mere capriciousness of epithelial cells in culture. Each of the cultured cell models employed in polarity studies derive from and reflect some of the differentiated features of a tissue or organ system. Accordingly, the sorting behavior observed in a particular cell type needs to be evaluated in the context of this cell's functional history. For example, is this cell derived from a tissue specialized for apical secretion or apical endocytosis? Studies of the sorting of ion-transporting ATPase molecules expressed in distal tubule-derived MDCK and proximal tubule derived-LLC-PK1 kidney cells suggest that the distinct cell surface distributions which an ATPase subunit achieve in these two lines are consistent with established physiologic differences between the distal and proximal tubule epithelial cells (Roush et al., manuscript submitted). These observations have led to the suggestion that sorting mediates delivery to functionally defined rather than topographically defined domains (Gottardi and Caplan, 1993a).

### **III. POLARIZED SORTING AND TARGETTING MACHINERY: ELEMENTS OF THE INTRACELLULAR PROTEIN TRANSPORT MACHINERY?**

It is becoming quite clear that the findings in the field of intracellular protein transport (reviewed by Rothman, 1994 and by Mellman, 1994) will prove to be extremely valuable to the discipline of epithelial polarity. In this field, the convergence of studies on synaptic vesicle (regulated) secretion in neurons, constitutive secretion in yeast, and intra-Golgi transport have led to the rapid identification and characterization of the basic components necessary for vesicle formation, docking and fusion. Clearly, the general components of the bud-

ding and docking machinery lie at the heart of any transport process, whether we are considering the transport of a membrane protein from ER to Golgi, or a secretory protein from the TGN to a particular cell surface. In the following sections we touch upon some of the key discoveries in the field of intracellular transport and focus on the relevant molecules that may contribute to polarized sorting and delivery processes.

### A. GTPases and Epithelial Polarity

One of the recent paradigms in intracellular protein transport is based on the concept that vesicle shuttling between different organellar compartments is regulated through the coordinated efforts of different GTP-binding proteins. There are two broad classes of GTP-binding proteins which have been shown to regulate membrane trafficking events; the small G proteins (rabs and ARF) reviewed by (Donaldson and Klausner, 1994; Pfeffer, 1992, 1994) and the trimeric G proteins (reviewed by Bomsel and Mostov, 1992).

### B. Rabs

The role of a GTP-binding protein in regulating vesicular transport was first realized with the analysis of one of the temperature sensitive SEC (secretory) mutants in yeast (Salminen and Novick, 1987). *Sec4* mutants display a rather striking accumulation of secretory vesicles when cultured at the restrictive temperature. The cloning, sequencing, and characterization of the *SEC4* gene revealed that it encoded a ras-like or 'small' GTP-binding protein which was present on the surfaces of the vesicles and could bind and hydrolyze GTP (Salminen and Novick, 1987; Goud et al., 1988; Kabcenell et al., 1990). Since the phenotype of cells bearing mutant *sec4* is the accumulation of transport vesicles, it was apparent that *SEC4* is necessary for the targeting and/or fusion of secretory vesicles with the plasma membrane. Similar results were found with another yeast protein YPT1 (48% identical to *SEC4*), which in its mutant form inhibited vesicular transport between the ER and Golgi complex (Gallwitz et al., 1983; Segev et al., 1988).

The suggestion that two small GTPases were important in the regulation of two different vesicular transport events in yeast led to the hypothesis that each step in vesicular traffic was regulated by a specific GTPase (Bourne et al., 1990). These ras-like GTPases are known to adopt either of two distinct conformations, depending upon whether or not they are complexed with GTP or GDP. Consequently, these GTPases have been postulated to serve as key regulators or "molecular switches" for membrane fission and fusion events. The apparent generality of ras-like GTPase in yeast, as revealed by *sec4* and *ypt1*, inspired a search for these proteins' mammalian counterparts. To date, 30 YPT1/SEC4-related proteins have been identified and are often referred to as rab proteins ("Ras-like" proteins from rat brain) reviewed in (Balch, 1990; Hall, 1990; Goud and McCaffrey, 1991; Zerial and Stenmark, 1994).

A number of rabs have been localized to specific organelles within the cell and through the combined efforts of *in vitro* and *in vivo* approaches have been shown to regulate membrane traffic between these organelles (reviewed in Zerial and Stenmark, 1994). How this class of molecules contributes to the overall fidelity of membrane trafficking events is still unclear (Rothman, 1994).

The idea that specific rab proteins regulate distinct steps along the transport pathway (e.g., rab1 always regulates ER to Golgi traffic, whether in a kidney cell or neuron) led to the hypothesis that cells which contain unique, cell-type specific transport processes might be regulated by distinct rabs. Indeed, the best example of this is the family of rab3 isoforms which have been found to be localized within cells which are well-adapted for regulated secretory events. Rab3a has been suggested to be important in the regulation of Ca<sup>2+</sup> dependent secretion in neuronal (Fischer von Mollard et al., 1991), neuroendocrine (Darchen et al., 1990) and endocrine cell types (Mizoguchi et al., 1989). Interestingly, an isoform of rab3a, rab3d, has been localized to the glucose transporter-containing vesicles of adipocytes, which are known to undergo regulated exocytosis after insulin stimulation (Baldini et al., 1992). Thus, despite cell-specific differences, or vesicle-content differences, these regulated pathways rely on similar rabs (rab3). Thus, distinct regulated exocytic events in a variety of cell types make use of similar molecular machinery (Lutcke et al., 1993).

In this context, it has been speculated that polarized epithelial cells, with their distinct apical and basolateral targeting pathways, may employ epithelia-specific rab molecules. Recent data suggest that this may be true. There are four rabs which have been implicated in polarized epithelial-specific functions: rab17, rab 3b, rab13, and rab8. Of the four, only rab17 is truly specific to polarized epithelial cells. In the developing kidney, rab17 mRNA is detected only after mesenchyme is induced to differentiate into polarized epithelial structures (Lutcke et al., 1993). Interestingly, rab17 induction was shown to occur just prior to the appearance of apical markers and has therefore been suggested to be involved in the generation of apical/basolateral polarity in these cells. Rab17 localizes to the basolateral membrane and to electron dense tubules near the apical membrane. Since rab proteins have been shown to regulate transport between the subcellular compartments with which they associate, it has been suggested that rab17 regulates epithelial transcytosis.

As we stated previously, two isoforms of rab3 (3A and 3D) have been implicated in the regulated exocytosis events shared by neuronal, endocrine and adipocyte cell types. Interestingly, another isoform of rab3, 3b, has been shown to be specific for polarized epithelial cells and is exclusively localized to the apical pole of cells, near the tight junctions (Weber et al., 1994). Rab13, like rab3b, also accumulates at the apical poles of polarized cells and co-localizes with the tight junction associated protein, ZO-1 (Zahraoui et al., 1994). It has been suggested that these two rabs could regulate events necessary for the establishment of polarity. For example, since the localization of both rabs are completely dependent on the presence the of cell-cell contacts, it is possible that these mole-

cules control the recruitment of membrane protein-containing vesicles required for establishing the tight junction "fence," a structure thought to maintain the distinct protein and lipid compositions of apical and basolateral membranes (Dragsten et al., 1981).

It has also been proposed that these rabs control general vesicle targeting to the apical membrane (Zahraoui et al., 1994). This hypothesis was based on two independent observations. It has been shown that an apical membrane protein (aminopeptidase) inserts preferentially into the apical membrane at regions of cell-cell contact in MDCK cells (Louvard, 1980). Furthermore, under conditions in which MDCK cells are denied intercellular contacts, apical proteins appear to be sorted and retained within a large subapical vacuolar compartment (vacuolar apical compartment, or VAC) which, after initiation of cell-cell contact, is inserted preferentially at regions of cell-cell contact (Vega-Salas et al., 1988). Taken together, the localization of rab13 and rab3b at this region of cell contact places these monomeric GTPases in a position to regulate the delivery of apical proteins to the cell surface (Zahraoui et al., 1994). Moreover, the localization of a regulated, exocytic compartment-specific rab (rab3) to a subdomain of the apical membrane of polarized cells is intriguing and suggests possible functional relationships between these sub-cellular compartments.

The last rab worth exploring in the context of epithelial polarity is rab8. While rab8 is not solely expressed in polarized cells, it is the only rab that has been functionally implicated in vectorial targeting. A peptide derived from the C-terminal region of rab8 can inhibit basolateral but not apical transport of membrane proteins in a permeabilized-MDCK cell assay (Huber et al., 1993a). Interestingly, rab8 can also regulate membrane transport to the dendritic plasma membranes of neurons in culture; antisense rab8 oligonucleotides decrease the level of viral glycoprotein transported to this domain (Huber et al., 1993b). This observation is consistent with the model which suggests that the mechanisms which produce axon/dendrite polarity in neurons may be similar to those involved in apical/basolateral polarity in epithelia (Simons et al., 1992).

Taken together, the identification of a polarized epithelia-specific rab (rab17), and the localization of other rabs to specific polarized epithelial domains (rab13 and rab3B, apical; rab8, basolateral) suggests that rabs may regulate specific pathways in polarized epithelial cells. For the epithelial cell biologist, the obvious question is, "What brings about the pathway-specific localizations of rab proteins in polarized epithelial cells?" It has been demonstrated that the carboxy-terminal regions of rab proteins are responsible for their unique cellular localizations (Chavrier et al., 1991). It has been suggested that organelle-specific receptors exist which recognize the C-terminal domains of these molecules. At least in terms of polarized cells, it would be tempting to speculate that identification of such receptors for rab13, 3b and rab8 will bring us one step closer to an understanding of the overall machinery that orchestrates domain-specific vesicle formation and targeting.



Recent evidence, however, suggests that rabs may not provide the primary level of specificity in membrane targeting events (Brennwald and Novick, 1993; reviewed by Rothman and Warren, 1994). As we discuss below, a new class of proteins, the SNARES, may provide the necessary specificity for vesicle–membrane targeting events throughout the cell.

### C. The SNARE Paradigm and Epithelial Polarity

The SNARE hypothesis for vesicle targeting arose from research in three related fields: synaptic vesicle release in neurons, transport between cisternae of the Golgi, and secretion in yeast. Briefly, a number of synaptic proteins were discovered to be important for the regulated fusion of synaptic vesicles with their targets on the pre-synaptic plasma membranes (reviewed by Pevsner and Scheller, 1994). Homologues of these proteins were found in yeast and shown to be required for constitutive vesicle transport (Aalto et al., 1993). At the same time, key elements of the general machinery for intracellular membrane fusion were being elucidated. In all three cases, membrane fusion requires an NEM-sensitive factor (NSF), adaptors that link NSF to membrane proteins (SNAPs: Soluble NSF Attachment Proteins) and the membrane receptors for the NSF-SNAP complexes (SNAREs: SNAP Receptors) (reviewed in Rothman and Warren, 1994). Distinct SNARE proteins are present in the membranes of the vesicle and the target. The SNARE hypothesis stipulates that each transport vesicle is endowed with its own vesicle- (*v*-) SNARE (or VAMP-like molecule) that can specifically interact with its cognate target- (*t*-) SNARE (or syntaxin/SNAP25-like protein). This ‘pairing’ could ensure vesicle/target membrane specificity, while a general fusion apparatus consisting of NSF and SNAPs could be used throughout the cell (Sollner et al., 1993).

In the context of epithelial polarity, this hypothesis suggests that vectorial targeting of apical and basolateral proteins will require distinct *v*-SNAREs. Interesting recent data suggest that the situation in at least one epithelial cell type may be somewhat more complicated. When the surface delivery of membrane proteins is examined in MDCK cells permeabilized at their apical or basolateral surfaces with streptolysin O, it appears that basolateral transport involves all of the machinery discussed above. Toxins which cleave SNAREs inhibit basolateral delivery, as do antibodies directed against SNAPs. In contrast, apical protein insertion is unaffected by these reagents. Isolation of apically-bound vesicles from MDCK cells reveals the presence of high concentrations of an adducin homologue in their surface membranes. Adducins are calcium-dependent phospholipid binding proteins thought to be involved in a number of membrane fusion events (Ilkonen et al., 1995). It would appear, therefore, that completely distinct classes of vesicular targeting and fusion machinery may operate in the two membrane delivery pathways present in polarized epithelial cells.

#### D. Insights from the Membrane Traffic-Perturbing Reagent, BFA

In the absence of a readily available genetic system with which to identify the genes and gene products necessary for such higher eukarotic functions as transcytosis or polarized targeting, epithelial cell biologists have been resigned to the prospect of “poking” at the epithelial cell with various reagents and watching how it responds. Reagents which prevent the polymerization of actin (Gottlieb et al., 1993; Jackmon et al., 1994) and tubulin (Achler et al., 1989; Parczyk et al., 1989), toxins which modify a particular class of G proteins (Stow et al., 1991; Pimplikar and Simons, 1993b), or toxins that inactivate the VAMP, syntaxin and SNAP-25 molecules described above, second messenger stimulators, analogues of the messengers themselves (Apodaca et al., 1994; Cardone et al., 1994; Hansen and Casanova, 1994) and the remarkable fungal metabolite brefeldin A (BFA) are all being incorporated into the repertoire of tools which we hope will enable us to glean more information from a particular transport pathway. Those interested in polarized and nonpolarized cell functions alike have made use of such cell-perturbing reagents. Since the focus of this review is epithelial polarity, we have chosen to summarize some of the studies which are providing insights about the mechanisms of polarized sorting and targeting.

#### E. BFA, Vesicle Bud Formation, and Polarized Trafficking Events

Brefeldin A is a fungal metabolite that endeared itself to cell biologists because of its dramatic effect on the protein secretory pathway (reviewed in Klausner et al., 1992). Protein secretion is inhibited by BFA: membrane trafficking out of the ER is blocked and the Golgi appears to breakdown and become redistributed into the ER (Lippincott-Schwartz et al., 1989). Before Golgi redistribution, BFA causes this organelle to form tubular extensions which are devoid of any cytoplasmic (non-clathrin) “coat” material (Lippincott-Schwartz et al., 1990). It has been shown that these morphological changes are not restricted to the Golgi but rather are observed in a number of organelles of the endomembranous network such as endosomes, lysosomes and the TGN (Hunziker et al., 1991; Lippincott-Schwartz et al., 1991; Wood et al., 1991), suggesting that the BFA “effector” might play a role in membrane transport events all over the cell. Perhaps surprisingly, while membrane transport phenomena are remarkably altered in the presence of BFA, several processes are clearly unaffected, including receptor mediated endocytosis and endocytic recycling (Lippincott-Schwartz et al., 1991).

From the standpoint of sorting and polarized delivery, BFA’s most interesting property is its ability to differentially affect polarized cell surface targeting events. For example, Low and colleagues (1991, 1992) determined a concentration of BFA where ER-Golgi trafficking was not inhibited, so that delivery from the TGN to the surface could be assayed for BFA sensitivity. Interestingly, BFA inhibited the apical delivery of both endogenous, MDCK secretory proteins (1991) and the membrane

protein DPPIV (1992) while also enhancing their mis-delivery to the basolateral surface. Basolateral targeting of the endogenous MDCK protein, uvomorulin, was not affected under these conditions. Taken together, it would seem that a target molecule for BFA action exists that is exclusively involved in directing apical vesicles or which is simply more sensitive to the effects of BFA than similar molecules participating in the basolateral pathway. Either way, these results provide a hint that there are indeed molecular differences between these two targeting pathways.

It is important to add that in addition to inhibiting the exocytic apical pathway in MDCK cells, basolateral to apical transcytosis is also inhibited by this drug (Hunziker et al., 1991; Low et al., 1992). These findings have led to the suggestion that sorting mechanisms for apically destined proteins, whether along the exocytic or the transcytotic pathway may be functionally and biochemically similar (Hunziker et al., 1991).

The fact that the loss of the structural integrity of the Golgi induced by BFA correlates with a striking absence of its characteristic "coat" (observed at the EM level) led to the idea that coat proteins might be rendered non-functional due to BFA action. Through a number of studies (reviewed by Donaldson et al., 1992; Helms and Rothman, 1992; Klausner et al., 1992; Rothman & Orci, 1992) molecules which make up this "coat" were identified and characterized (e.g.,  $\beta$ COP and ARF). An "order of events" necessary for vesicle budding emerged from these studies and is outlined below. ARF is a GTP-binding protein loosely related to ras and distinct from the family of rabs. In its GTP-bound state, it is capable of associating with the membrane by virtue of its myristoyl group, while its GDP-bound form is soluble and not membrane bound. ARF binding to membranes appears to be the signal for coatamer binding, that is, the binding of  $\beta$ COP in addition to other as yet uncharacterized coat proteins. Coatamer binding is believed to be absolutely necessary for vesicle budding. Therefore proper coatamer assembly would be required for any event downstream of budding, such as targeting. Recently, it has been determined that BFA inhibits coatamer assembly and vesicle formation through ARF, by essentially allowing it to remain in its GDP-bound or inactive form. There exists a class of proteins which are able to catalyze the exchange of GDP for GTP called guanine nucleotide exchange factors (GNE). BFA has been proposed to antagonize the action of a GNE on ARF, thus preventing coatamer assembly and membrane budding (Donaldson et al., 1992; Helms and Rothman, 1992).

With the recent identification of an ever-growing family of new ARF-related genes (Kahn et al., 1991) and the speculation that different COPs may exist in the control of membrane budding events from different organelles (Matter and Mellman, 1994), there is growing excitement that ARFs and COPs will turn-out to be essential components for regulating a particular level of specificity inherent to membrane targeting events. In the context of BFA's affect on apical sorting and targeting in polarized MDCK cells (Low et al., 1991, 1992), it is likely that distinct ARF/coatamer complexes regulate the budding of apical and basolaterally-destined vesicles from the TGN. Moreover, the fact that significant missorting into

the basolateral pathway was observed in the presence of BFA (Low et al., 1992) suggests that coatamer assembly may be inextricably linked to proper secretory and membrane protein sorting.

#### F. Heterotrimeric G Proteins and Sorting

It has been known for some time that members of the heterotrimeric family of G proteins are associated not only with the plasma membrane but also with intracellular membranes (reviewed by Bomsel and Mostov, 1992). A number of toxins (cholera, pertussis and mastoparan) known to activate or inhibit various classes of G proteins have been applied to studies of polarized sorting and targeting. Stow et al. (1991) found that overexpression of  $G\alpha_i-3$  in polarized LLC-PK1 cells significantly reduced the level of constitutive basolateral secretion of an extracellular matrix component, heparan sulfate proteoglycan. Pertussis toxin, which ADP-ribosylates and inactivates the  $\alpha$ -subunits of the  $G\alpha_{i/o}$  class of heterotrimeric G proteins, relieved this inhibition. Similarly, Pimplikar and Simons (1993) suggested that  $G_i$  and  $G_s$  may differentially regulate the trafficking of apical and basolateral vesicles in SLO-permeabilized MDCK cells, while Leyte et al. (1992) found that  $G_{i/o}$  and  $G_s$  associated with the TGN could oppositely regulate constitutive secretory vesicle formation. It should be noted that in no case did the G protein related inhibition or stimulation appear to affect the actual sorting or missorting of apical or basolaterally destined proteins (in contrast to the BFA results discussed above (Low et al., 1992), but rather may only affect the rate or "efficiency" of sorting/targetting (Pimplikar and Simons, 1993a).

A possible link between heterotrimeric G proteins and coatamer formation/vesicle budding was provided by Ktistakis et al., (1992). This group found that activation of a  $G\alpha$  protein with mastoparan promoted  $\beta$ COP binding and prevented BFA-induced effects. Pretreatment of cells with pertussis toxin, which is known to specifically affect  $G\alpha_i$  subclass of heterotrimers, prevented mastoparan's antagonizing effects on BFA. Stated more simply, these results showed that activation of a pertussis-toxin-sensitive  $G\alpha$  promotes the binding of  $\beta$ COP to Golgi membranes and thus antagonizes the action of BFA. The authors of this study suggest further that different subclasses or isoforms of  $G\alpha$  could be responsible for some of the differences in BFA-sensitivities observed between cell types and organellar membranes.

These key observations have led to the idea that heterotrimeric G proteins, by virtue of their membrane topology would be ideal candidates for coordinating the transfer of sorting information to the cytoplasmic surface of the TGN necessary for vesicle budding (Bomsel and Mostov, 1992; Ktistakis et al., 1992).

#### G. Insights from Genetic Models

The outer surface of a fruit fly embryo is composed of a monolayer of polarized epithelial cells. The apical membranes of these epithelial cells face the outer shell,

or chorion, while their basolateral surfaces face the embryonic interior and yolk space. Invaginations of this surface epithelium give rise to all of the embryo's internal tissue structures (for review see Shiel and Caplan, 1995b). Recent investigations have examined the mechanisms through which proteins are sorted in these epithelial cells.

Human placental alkaline phosphatase (PLAP) is a GPI-linked protein which has been shown to be sorted to the apical plasma membrane when it is expressed by transfection in MDCK cells. A chimeric construct of PLAP, in which the GPI-linkage domain is replaced by the transmembrane and cytoplasmic domains of the VSV G protein (PLAPG), is sorted to the basolateral surfaces of MDCK cells (Brown et al., 1989). These two proteins have been expressed under the control of heat shock promoters in transgenic flies and their distributions have been examined in embryonic epithelia throughout embryogenesis (Shiel and Caplan, 1995a). As would be expected, the PLAPG protein is restricted to basolateral surfaces throughout ontogeny in the surface epithelial cells as well as in the internal epithelia which derive from invaginations of the surface cells.

Surprisingly, PLAP was also restricted to a basolateral distribution in the surface epithelial cells in both early and late stage embryos. Biochemical experiments demonstrated that this mis-sorting of the PLAP protein can not be attributed to problems with the addition of the GPI-linkage, since at all embryonic stages PLAP is correctly glycosylated. Internal epithelial cells sorted PLAP exclusively to their apical surfaces. Since in many cases internal epithelia form from surface epithelia without undergoing any mitosis (e.g., salivary gland), essentially the same epithelial cell is capable of differentially sorting PLAP depending on that cell's physical position within the embryo. Examination of epithelia undergoing invagination (e.g., ventral furrow, tracheal placode) demonstrate that the transition in PLAP sorting occurs in the early stages of the invagination process. While the mechanism responsible for this switch remains unclear, the power of *Drosophila* genetics will hopefully allow the cellular components responsible for this transition to be readily identified. It is likely that the isolation of the proteins responsible for this phenomenon will shed light on the *Drosophila* as well as on the mammalian epithelial sorting machinery.

A *Drosophila* mutation whose phenotype includes perturbations of the polarized organization of the surface epithelial cells has recently been identified and characterized at the molecular level (Knust et al., 1993; Wodarz et al., 1993). The crumbs gene encodes a transmembrane protein which is normally expressed in the apical membranes of surface and internal epithelial cells. Mutation of the crumbs gene results in a loss of crumbs polarity and markedly alters embryonic morphology. Genetic studies have demonstrated that the crumbs gene product is necessary not only for its own apical sorting, but for the apical delivery of other proteins as well. Furthermore, the product of the stardust gene appears to interact with the crumbs protein and also appears to participate in apical sorting. Understanding these proteins' biochemical functions and their intermolecular associations will undoubtedly provide enormous insight into the cellular components responsible for generating and

maintaining the polarized phenotype. Hopefully, the development of genetic approaches such as these, in concert with the continuing refinement of *in vitro* and model systems, will allow us to develop a clear and fundamental understanding of how epithelial cells produce their remarkable asymmetry.

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