CHAPTER 2

Biogenesis and Sorting of Plasma Membrane Proteins

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I. INTRODUCTION

The plasma membrane constitutes an animal cell's link with and protection from the outside world. The cell surface membrane serves as the selective permeability barrier which supports the nonequilibrium distribution of solutes necessary for the maintenance of homeostasis. The plasmalemma also possesses the elements which endow a cell with the capacity to converse with its environment. It is now widely accepted that

the membrane's protein components deserve most of the credit for these manifold capabilities. As is discussed throughout this volume, it is channel, pump, and cotransport proteins embedded in the cell surface lipid bilayer which control the composition of the intracellular milieu. Plasmalemmal receptor and transducer proteins allow the cell to recognize and respond to various external influences. Membrane-associated proteins anchor cells to their substrata and mediate their integration into tissues. These examples suggest that many of the properties of a given cell type may be attributed to the protein composition of its plasma membrane (Guidotti, 1986). It is not surprising, therefore, that most cells go to great lengths to control the nature and distribution of polypeptides which populate their plasmalemmas.

There are a number of ways in which the cell can exert this control. As is true for almost any polypeptide, cells carefully regulate the expression of genes encoding plasma membrane proteins (Darnell, 1982). Transcriptional regulation probably accounts for most of the individuality associated with the cell surface membrane. The extent to which the plasmalemmal constituents of two cells differ from one another probably reflects the idiosyncracies of their respective genetic programs more than any other single influence. Posttranscriptional control of synthesis may also play a role in defining the composition of the plasma membrane. Recent evidence from a number of systems suggests that the cell may not diligently apply itself to translating each and every mRNA as soon as it is transcribed. Mechanisms exist through which messenger RNAs (especially those specifying secretory and membrane proteins) can be warehoused in the cytosol until the proteins which they encode are called for (Walter and Blobel, 1981b; Wolin and Walter, 1988, 1989).

Although transcriptional and translational controls are extremely important, the cell's ability to oversee the assembly of its plasmalemma does not end with protein synthesis. Proteins destined for insertion into the plasma membrane pass through a complex system of processing organelles prior to arriving at their site of ultimate functional residence. Each of these organelles makes a unique contribution to the maturation of these proteins as they transit through them. Each of these organelles also serves as a potential decision point, at which the cell can make choices about a protein's delivery, distribution, and life span.

This chapter focuses on the postsynthetic steps involved in the biogenesis of plasma membrane proteins. It begins with a discussion of some of the events common to all plasmalemmal polypeptides, with special emphasis on those which contribute directly to the character of the cell surface. The second half of the chapter is devoted to the specializations associated with cell types possessing differentiated cell surface sub-

domains. Epithelial cells (among many other cell types) are characterized by plasma membranes which can be morphologically and functionally divided into two or more distinct compartments (Simons and Fuller, 1985; Matlin, 1986; Caplan and Matlin, 1989; Rodriguez-Boulan and Nelson, 1989). The pathways through which membrane proteins are sorted to and retained in these differentiated regions of the cell surface are just beginning to be understood.

The mechanisms through which the plasma membrane is generated and maintained have bearing on all of the processes discussed in this volume. Our current understanding of these mechanisms is, for the most part, rudimentary and rapidly evolving. The goal of the synopsis presented here, therefore, is to not to provide an exhaustive review. Rather, its aim is to acquaint its readers with some of the important and fascinating questions confronting investigators interested in the cell biology of the plasma membrane.

II. BIOGENESIS OF MEMBRANE PROTEINS

A. *The Processing Pathway*

In the late 1960s, Jamieson and Palade demonstrated that newly synthesized secretory proteins follow a rigorously prescribed route through the cell (Jamieson and Palade, 1967a,b, 1968a,b; Palade, 1975). Autoradiographic experiments at the electron microscopic level revealed that, on completion of their synthesis, the zymogens of pancreatic exocrine cells are first associated with the rough endoplasmic reticulum (RER). Several minutes later, the newly synthesized polypeptides are transported, via vesicular carriers, to the Golgi complex. The newly synthesized proteins are delivered to the cis-most (i.e., ER-facing) stack of the Golgi and subsequently progress through each stack to the terminal or trans-most cisterna. Condensation of the secretory proteins begins in the trans Golgi stack and continues as the zymogens pass through condensing vacuoles to secretory granules, where they remain until they are discharged from the cell in response to a secretagogue. Although aspects of this pathway reflect specializations unique to endocrine and exocrine secretory cells, in general outline it has proved applicable to virtually any type of animal cell. Furthermore (and most importantly for this discussion), the pathway delineated in these studies is also pursued by newly synthesized membrane proteins (Bergmann and Singer, 1983).

Over the ensuing years it has been shown that each step along the route which a membrane protein follows to the cell surface, i.e., ER, cis Golgi,

trans Golgi, is associated with specific processing reactions. Thus, the postsynthetic maturation of secretory and membrane proteins is temporally and spatially compartmentalized. The reactions which occur in each organelle build on those which were carried out at the previous station. Furthermore, the processing steps associated with a given locus are frequently absolute prerequisites for the further processing events which will occur in subsequent locations. With regard to these features, the post-translational course pursued by membrane and secretory proteins is analogous to an assembly line. Like an assembly line, the process is carried out vectorially and in strict series. There is no evidence for skipping or repeating steps under normal circumstances. The flux of proteins from the ER to the cell surface is, for the most part, unidirectional (Palade, 1975; Rothman, 1987). Each step of the process is also endowed with some capacity for quality control. The cell can monitor the degree to which processing steps have been successfully completed and, if it is not satisfied, it can activate mechanisms to detain or destroy those proteins which it judges to be imperfect (Lippincott-Schwartz, 1988).

Successful passage through this endomembraneous network is required of every protein which will ultimately reside in the plasmalemma. Our understanding of the mechanism and purpose underlying each of the modifications associated with stations along this route is far from complete. It is clear, however, that the biosynthetic pathway contributes a number of possible points at which control of the composition of cell surface membrane can be exerted. The sections which follow describe the steps which constitute this pathway in greater detail.

B. The Role of the Endoplasmic Reticulum: Membrane Insertion

The machinery responsible for protein synthesis resides in the cytoplasm (Palade, 1975). Ribosomes, tRNAs, mRNAs, elongation factors, etc. are all soluble components of the cytoplasmic space. In synthesizing a membrane or secretory protein, therefore, the cell is faced with the problem of producing a polypeptide which must ultimately be separated by a lipid bilayer from the molecules required for its production. The pathways which cells have evolved to overcome this difficulty have been explored during the course of over two decades of elegant research.

It is now generally accepted that the vast majority of animal cell membrane and secretory proteins are inserted into or across the membrane of the RER cotranslationally (Walter and Lingappa, 1986). Ribosomes translating secretory or membrane proteins become bound to the cytoplasmic

surface of the ER. The polypeptide emerging from the bound ribosome enters and traverses the ER membrane as it is elongated (Blobel and Dobberstein, 1975). When translation is complete, the protein is released from the ribosome and the ribosome is released from the RER membrane. In the case of secretory proteins, release from the ribosome results in the discharge of the polypeptide across the membrane and into the lumen of the RER. Transmembrane proteins are released in what will presumably be their final topology, with endodomains facing the cytosol, ectodomains exposed at the lumenal face of the RER, and membrane-spanning domains embedded in the lipid bilayer (Blobel, 1980).

Proteins destined for cotranslational insertion into or across the RER generally carry within their primary structure a contiguous stretch of ~ 15 amino acids which is required to initiate the membrane interaction. These "signal sequences" tend to be fairly hydrophobic and are most commonly located at a protein's amino terminus (von Heijne, 1985). Upon emerging from the ribosome, the signal sequence interacts specifically with a cytosolic complex of proteins and RNA called signal recognition particle (SRP) (Walter and Blobel, 1981a,b; Walter et al., 1981).

The interaction with SRP serves two purposes. Evidence from *in vitro* studies indicates that association with SRP often causes a temporary translation arrest (Walter and Blobel, 1981b; Wolin and Walter, 1989). The nascent polypeptide remains bound to the polysome, but elongation ceases. The bound SRP also acts as a guide which directs a polysome to the membrane of the RER. Targeting of the SRP–polysome complex to the RER requires the intercession of the SRP receptor or docking protein, a heterodimeric transmembrane component of the RER which acts as a receptor for SRT-bearing polysomes (Meyer et al., 1982; Gilmore et al., 1982a,b). Interestingly, one of the polypeptides of the SRP receptor shares a region of homology with one of the protein components of SRP itself, prompting the speculation that this domain corresponds to a signal sequence binding site (Bernstein et al., 1989; Romisch et al., 1989).

Once bound to docking protein, the SRP–polysome complex dissociates. Another transmembrane component of the RER appears to bind the signal sequence after its release from SRP (Weidmann et al., 1987). Discharge of SRP from the ribosome requires GTP, which appears to interact with the docking protein (Connolly and Gilmore, 1989; Hoffman and Gilmore, 1988) and perhaps with the 54-kDa subunit of SRP itself (Bernstein et al., 1989; Romisch et al., 1989). On release of SRP, the translation block is relieved and synthesis of the nascent polypeptide continues in association with the (as yet poorly understood) machinery responsible for actually translocating elongating polypeptides across the ER membrane (Krieg et al., 1989). This translocation is not driven by translation and seems to

require energy in the form of hydrolyzable nucleotides (Perara *et al.*, 1986; Chen and Tai, 1987; Hoffman and Gilmore, 1988). By halting translation until a free docking protein site on the RER is encountered, SRP may prevent secretory and membrane proteins from being completely synthesized and discharged into the cytosol. If no translocation sites on the RER are available, SRP may serve to route the translation complex into what might be characterized as a holding pattern. Resumption of active translation is predicated upon the translocation machinery signaling its readiness to process one of those waiting in line.

Once it has succeeded in initiating translocation, the signal sequence is of no further use. Signal peptidase, an enzyme resident in the membrane of the RER, cleaves most amino-terminal signal sequences from their parent proteins, leaving the mature polypeptide ~ 15 amino acids shorter than the protein encoded by the mRNA (Evans *et al.*, 1986). Interestingly, it is precisely this cotranslational removal of the signal sequence which greatly facilitated the discovery and characterization of the signal sequence-mediated membrane insertion pathway (Milstein *et al.*, 1972).

It must be pointed out that the pathway described above, although elegant, is not all encompassing. A large number of deviations from this model have been documented. Most dramatic of these, perhaps, is the situation which prevails for proteins inserted into the membranes of mitochondria and chloroplasts. It is now clear that these polypeptides are synthesized in their entirety in the cytosol (Attardi and Schatz, 1988; Schmidt and Mishkind, 1986). Following completion of their translation, they undergo signal sequence-mediated import into or across the relevant membrane. Posttranslational membrane insertion has also been demonstrated in yeast and bacteria (Hansen *et al.*, 1986; Waters and Blobel, 1986; Rothblatt and Meyer, 1986; Wickner, 1988). The situation in yeast is especially interesting, since these organisms possess an endomembranous system which is extremely similar to that found in higher eukaryotes. Yeast secretory and membrane proteins can be synthesized on free, cytoplasmic ribosomes and discharged into the cytoplasm as soluble proteins. In order to retain the ability to be inserted into or across the membrane of the ER, however, these newly synthesized polypeptides must associate with members of a class of cytosolic proteins which serve to prevent them from folding into their final conformations. In bacteria the GroEL family of proteins serves this purpose (Hemingson *et al.*, 1988), whereas in yeast the hsc70 complex mediates this function (Deshaies *et al.*, 1988; Chirico *et al.*, 1988). These "chaperonins," which are related to the heat-shock family, serve a function somewhat analogous to that of SRP. Their associations with newly synthesized proteins maintain the competence of these polypeptides for membrane insertion until this opera-

tion can be accomplished. Dissociation of the unfolded protein from the chaperonin is energy dependent (Rothman, 1989).

Signal sequences need not always be amino terminal. It is becoming clear that stretches of hydrophobic amino acids can serve as signal sequences when placed at a number of different positions in a protein's linear sequence. Although this phenomenon has been demonstrated for secretory proteins (Lingappa et al., 1979; Perara and Lingappa, 1985), it appears to be far more common among membrane proteins. It is now apparent that the hydrophobic membrane-spanning sequences which anchor proteins in the bilayer can also serve as their "start" or "stop" transfer sequences (Yost et al., 1983; Rothman et al., 1988). A large number (perhaps even the majority) of membrane proteins have no amino-terminal signal peptide. This is especially true of polytopic proteins, that is, those which span the bilayer several times. The transmembrane domains of these multiloop proteins can apparently serve to start or stop translocation, thus mediating the SRP-dependent and cotranslational weaving of the nascent polypeptide into the membrane fabric of the RER (Anderson et al., 1983). The extent to which the interaction of transmembrane domains with SRP can cause translation arrest remains to be established.

The paradigm presented here for the insertion of membrane proteins into the RER is very much an evolving one. The component parts of the translocation machinery are just beginning to be identified (Krieg et al., 1989) and the biophysical parameters governing the insertion of a protein into or through a lipid bilayer remain mysterious (Engelman and Steitz, 1981). Furthermore, exceptions to this general rule continue to surface. Of special relevance to the topics discussed in this volume is the case of the erythrocyte glucose transporter. Recent evidence from in vitro translation experiments indicates that this protein, which spans the bilayer as many as 12 times, can be synthesized in its entirety on free cytosolic ribosomes and inserted into microsomes derived from the RER posttranslationally (Mueckler and Lodish, 1986). This behavior, although apparently commonplace in yeast and bacteria, is unprecedented among the plasmalemmal proteins of higher eukaryotes. It is not yet clear whether the glucose transporter is actually intercalated posttranslationally in vivo. The degree to which a chaperonin (related to the bacterial GroEL or yeast hsc70 class of proteins) might participate in this posttranslational insertion has also not been investigated. This is at least a possibility, since members of the chaperonin family have been shown to be present in the cytoplasm of mammalian cells. For instance, hsc70 is present in the cytoplasm of animal cells, where it is known to mediate the uncoating of clathrin-coated vesicles (Chappell et al., 1986). The prevalence of the glucose transporter's renegade conduct—among other transporters or other membrane proteins

in general—needs to be further investigated. Independent of its applicability, however, the example of the glucose transporter echoes a theme which has recurred several times during the preceding discussion: that the initial step in the biosynthesis of a plasma membrane protein is complex and offers the cell several options for variation and regulation.

Incorporation into the membrane of the RER constitutes the first in a series of sorting steps which will be encountered by a newly synthesized plasma membrane protein. This first step separates all membrane proteins from the rest of the polypeptides being synthesized coincidently. In a sense, the RER is a staging area, in which membrane proteins destined for a number of different subcellular locales are gathered in order to begin a course of common processing operations. Subsequent sorting events will separate these membrane proteins into subclasses destined for different destinations or modifications.

C. The Role of the Endoplasmic Reticulum: Processing and Transport to the Golgi Complex

1. Cotranslational Processing

The processing steps which contribute to the maturation of a plasmalemmal protein begin as soon as its membrane insertion is initiated. As portions of the protein are woven into and through the membrane, they undergo covalent modifications and start to assume a tertiary structure. In addition to cleavage of the signal sequence (Evans *et al.*, 1986), alterations generally associated with a membrane protein's residence in the ER include folding (Hurtley and Helenius, 1989), disulfide bond formation (Freedman, 1989), N-linked glycosylation (Hirschberg and Snider, 1987), and oligomerization (Hurtley and Helenius, 1989). Departure from the RER frequently requires that these operations be successfully completed (Rose and Doms, 1988).

A great deal has been learned about the biochemical processes involved in N-linked glycosylation. Although the purposes served by the addition of sugar groups to proteins remain rather mysterious, the mechanics of their addition are well worked out. The sugar structure characteristic of N-linked glycosylation is a branched tree containing (among other sugar types) nine mannose residues and an *N*-acetylglucosamine at its stem (Lennarz, 1987). While it is being preassembled in the ER, the sugar structure is attached to the membrane through a linkage between its initial *N*-acetylglucosamine residue and a molecule of dolichol phosphate lipid. The completed sugar tree is transfered en bloc from the dolichol lipid to lumenally disposed asparagine acceptor sites on nascent polypeptides.

Addition of the sugar occurs while the proteins are still in the process of being cotranslationally inserted into and across the bilayer. Sugars are added only to those asparagine residues which appear in the appropriate context, that is, as part of the sequence Asn-X-Ser or Asn-X-Thr. In many proteins, only a subset of those asparagines appearing in this configuration actually get glycosylated. The basis for this selectivity is not fully understood.

Disulfide bond formation is the responsibility of the lumenally disposed enzyme protein disulfide isomerase (PDI) (Freedman, 1989). As with glycosylation, disulfide bond formation proceeds cotranslationally. The PDI complex may actually be involved in several functions, including aspects of N-linked glycosylation.

On emerging into the lumen of the RER, the nascent polypeptide begins immediately to fold and acquire tertiary structure. This appears to be a complicated and multifactorial process, involving both the biophysical properties of the peptide chain itself and the intervention of protein complexes which catalyze folding. It has been elegantly demonstrated that, in mitochondria, for example, the folding of proteins delivered to the matrix space is controlled through an interaction with hsc60 (Ostermann *et al.*, 1989). This protein complex, composed of 14 identical subunits, is very similar in structure and function to the bacterial GroEl family (Hemingson *et al.*, 1988). These "foldases" appear to interact with nascent polypeptides as they traverse membranes and, through controlled binding and unbinding, play a role in orchestrating their folding (Rothman, 1989). It has been suggested that immunoglobin binding protein (see below) might serve a similar function for proteins inserted into the RER.

Finally, it must be noted that the addition of sugars and the formation of disulfide bonds will affect the balance of biophysical forces and thus contribute to the realization of the protein's ultimate conformation. Similarly, the acquisition of structure driven by the chemical nature of the primary sequence will influence the placement of sugars and disulfide bonds by making certain asparagine and cysteine residues more or less available to their respective modifying enzymes. Thus, a membrane protein's final tertiary conformation is formed as the result of a dynamic interplay among the proteins which facilitate folding, and the enzymatic reactions and biophysical forces which are brought to bear during cotranslational membrane insertion.

2. Oligomeric Assembly

Many plasmamembrane proteins are multimeric. This is certainly true of ion transporters, as exemplified by band III (a homodimer) (Jay and Cantley, 1986), the Na^+/K^+-ATPase (a heterodimer) (Jorgensen, 1982),

and the nicotinic acetylcholine receptor (a heteropentamer) (Popot and Changeux, 1984). Recent evidence indicates that assembly of newly synthesized monomers into the appropriate higher order structures occurs during their period of residence in the RER.

A number of studies illustrating this point have been performed on the trimeric spike glycoproteins of enveloped viruses. Studies combining pulse-chase labeling with density gradient centrifugation reveal that assembly occurs very early after the completion of protein synthesis (Copeland *et al.*, 1986, 1988; Gething *et al.*, 1986). These experiments also indicate that those monomers which do not get incorporated into trimers never pass from the ER to the Golgi. Temperature-sensitive mutants of spike glycoproteins which are barred from trimerizing at elevated temperatures can only depart the ER at a lower, permissive temperature (Doms *et al.*, 1988).

Retention in the ER of incompletely folded or assembled polypeptides may perhaps be mediated by a protein called immunoglobin binding protein (BiP) (Bole *et al.*, 1986; Hurtley *et al.*, 1989). This 72-kDa polypeptide is a soluble component of the ER lumenal space. It is a member of the family of glucose-regulated proteins, which are a subset of polypeptides encoded by the heat-shock response genes (Munro and Pelham, 1986). BiP binds to newly synthesized proteins whose folding or oligomerization is incomplete or has not been completed correctly (Hurtley and Helenius, 1989; Kassenbrock *et al.*, 1988). As was suggested above, an interaction with BiP may facilitate the folding of ER inserted proteins. BiP binding is apparently reversible and, at least *in vitro*, can be terminated through an interaction with ATP. The BiP polypeptide is itself retained in the ER by virtue of its four carboxy-terminal amino acids, whose sequence is KDEL (Munro and Pelham, 1987). It has been shown that proteins bearing this sequence at their carboxy termini are retained in the ER through an interaction with a recently identified lumenally disposed receptor (Vaux *et al.*, 1990). It has been suggested that this retention is active, that is, that KDEL-bearing proteins are capable of escaping to the Golgi or a pre-Golgi compartment, from which they are recaptured via a KDEL-mediated mechanism (Pelham, 1988).

Many misfolded or unassembled proteins retained in the ER are ultimately degraded in a compartment distinct both from the ER and the lysosome. This degradation has been best studied for the case of the T cell antigen receptor, which is composed of seven subunits and which apparently assembles inefficiently under normal circumstances (Clevers *et al.*, 1988). Only about 5% of this protein complex passes from the ER to the Golgi, while the remaining 95% is rapidly turned over by some process which cannot be interrupted by inhibitors of lysosomal enzymes (Klausner

et al., 1990). The nature and subcellular location of this degredative activity have yet to be established.

Similar observations on oligomeric assembly have been gathered for endogenous cellular proteins involved in membrane transport. Studies on the Na^+/K^+-ATPase indicate that the assembly of the α- and β-subunits to form the heterodimer occurs almost as soon as the proteins are released from the ribosomes (Tamkun and Fambrough, 1986). Studies in which the subunit polypeptides are expressed individually in fibroblasts (Takeyasu *et al.*, 1987, 1988) or in *Xenopus* oocytes (Geering *et al.*, 1989; Caplan, 1990) suggest that the α-subunit cannot leave the ER unless it is appropriately complexed with a β-subunit. These observations are especially interesting in light of the potential mechanism they offer the cell for regulating cell surface Na/K^+-ATPase levels. It has recently been shown, for example, that in chick myocytes the α-subunit is produced in excess of the β polypeptide (Taormino and Fambrough, 1990). Those α-subunits unable to assemble remain in the ER and are probably degraded through mechanisms similar to those discussed above. Conditions which induce the cell to increase its complement of cell surface sodium pump result in an increase in the transcription of only the mRNA encoding the β-subunit. The resultant increase in the quantity of newly synthesized β-subunit polypeptide in the membrane of the RER allows more heterodimer to assemble and more Na^+/K^+-ATPase to be delivered to the cell surface.

In the case of the Na^+/K^+-ATPase, time spent in the ER may effect more than folding and oligomerization. Recent evidence suggests that at least some aspects of the sodium pump's catalytic activity may be initiated during this early postsynthetic period (Caplan *et al.*, 1990; Geering *et al.*, 1987). Ouabain is a specific inhibitor of the Na^+/K^+-ATPase, which achieves its inhibition by binding to the sodium pump's α-subunit. Ouabain binds with greatest affinity to only one of the conformations through which the Na^+/K^+-ATPase passes during the course of its enzymatic cycle (Forbush, 1983). *In vitro,* the sodium pump can be driven into this conformation in two ways, one of which requires enzymatic hydrolysis of ATP and one of which does not. Experiments employing the photoactivatible NAB derivative of ouabain and anti-ouabain antibodies were performed on cultured kidney cells which had been briefly pulse labeled with {^{35}S]methionine (Caplan *et al.*, 1990). It was found that, immediately upon completion of the pulse period, newly synthesized radiolabeled Na^+/K^+-ATPase was capable of binding ouabain under the ATP-independent conditions. Ouabain binding requiring ATP hydrolysis, however, was not observed until the newly synthesized sodium pumps were at least 10 min old. Results from other studies suggest that the newly synthesized sodium pump still resides in the ER at this early time point (Tamkun and Fam-

brough, 1986). These observations suggest that the sodium pump may undergo some form of activation or conformational maturation during its passage through the ER. Similar conclusions have been generated in studies using controlled proteolysis to examine the conformational repertoire of the newly synthesized sodium pump (Geering *et al.*, 1987). The nature of this putative activation step remains to be elucidated. It is also not clear whether other ion pumps or, more generally, other plasma membrane enzymes undergo some manner of activation during the course of their postsynthetic processing in the ER. It is interesting to speculate, however, that intracellular activation might play a role in regulating the cell's plasmalemmal levels of functional Na^+/K^+-ATPase.

3. Transport to the Golgi

Newly synthesized proteins depart from the RER in vesicular carriers which bud from the ER membrane. Morphological evidence suggests that budding occurs from the transitional elements, specialized smooth subdomains of the RER which are closely apposed to the cis-most cisterna of a Golgi complex (Jamieson and Palade, 1967a). The vesicles appear to be coated with some matrix which is morphologically and immunologically distinct from clathrin (Orci *et al.*, 1986; Pfeffer and Rothman, 1987). Delivery of proteins to the Golgi can be reversibly blocked by incubating cells at 16°C (Saraste *et al.*, 1986). The formation of the vesicles and their subsequent targeting to the Golgi appears to be a complex, multistage process which has recently become susceptible to *in vitro* analysis. By examining the processing of newly synthesized membrane proteins in permeabilized cells, investigators have been able to define some of the soluble, cytosolic components which are necessary for the maintenance of ER to Golgi vesicular traffic. Once the vesicles have budded, GTP is required to activate them for the fusion step with the Golgi acceptor (Beckers and Balch, 1989; Beckers *et al.*, 1989). A cytosolic protein called NSF (*N*-ethylmaleimide sensitive factor) is also required at this stage. A cytosolic calcium concentration in the range of 0.1 μM appears to be critically required for fusion of the vesicular carrier with the cis Golgi stack.

The precise roles of these soluble effectors have yet to be defined. It is interesting to note, however, that GTP and NSF may be common elements in all of the subsequent vesicular transport events involved in carrying proteins to the plasmalemma. NSF has been isolated and found to be identical with an NEM-sensitive protein necessary for the movement of vesicles between succesive Golgi stacks (Beckers *et al.*, 1989; Malhorta *et al.*, 1988). GTP and small, ras-like GTP-binding proteins appear to be involved in each stage of the secretory pathway (Bourne, 1988), as is

discussed below. It is rather aesthetically pleasing to consider that the cell might exploit similar mechanisms to accomplish the transport of vesicular carriers between the numerous organelles in the secretory pathway. The mechanical aspects of budding and fusing vesicles are probably quite similar whether these operations are occurring in the ER or in the Golgi. It would not be surprising, therefore, if a considerable amount of the cellular machinery which has evolved to solve these problems in one setting were to be brought to bear in another. The extent to which this generalized machinery exists remains to established, as do the identities of components which could endow specificity to each pathway served by this putative general scheme.

Finally, it must be pointed out that the existence of vesicular flux from the ER to the Golgi suggests the existence of an equal flux of membrane from the Golgi to the ER. The idea that membrane delivered from the ER to Golgi must be retrieved arises from steady-state considerations and from the observation that the biochemical compositions of the ER and Golgi are discrete (Palade, 1975; Pfeffer and Rothman, 1987). Although it has long been postulated, evidence for this return traffic has only recently been gathered in experiments employing brefeldin A. This drug results in a dissolution of the Golgi complex and the redistribution of its membrane protein markers to the ER (Lippincott-Schwartz *et al.*, 1990; Doms *et al.*, 1989). In order to understand these observations, it has been suggested that a flux of vesicles normally carries membrane from the Golgi to the ER and that this flux is able to exclude, via some sorting process, polypeptides native to the Golgi cisternae. It is thought that brefeldin A may disturb this putative sorting process, thus allowing proteins normally associated exclusively with the Golgi membranes to enter the back-flux and thus become associated with the ER. According to this interpretation, the Golgi proteins which arrive in the ER are simply allowed to enter a pathway which normally exists in the cell and from which they are normally excluded. The validity of this assertion remains to be established. Some manifestation of the sorting mechanism which it postulates must exist, however, since newly synthesized membrane proteins pass from ER to Golgi to the cell surface, while the resident proteins of these compartments are not swept along in this flow.

D. The Role of the Golgi Complex: Covalent Modifications and Stabilization of Conformation

1. Sugar Modifications

The best studied functions associated with the membranous stacks of the Golgi complex relate to the processing of *N*-linked oligosaccharides. The sugar structure transferred from dolichol phosphate to the asparagine

residues of nascent polypeptides contains nine mannose and three glucose residues (Hirschberg and Snider, 1987). The glucose residues and one mannose residue are removed very shortly after the sugar chain is transferred from its dolichol intermediate to the acceptor polypeptide. This initial step in the pathway of sugar processing reactions occurs while the newly synthesized protein is still resident in the ER. All subsequent modifications are carried out by enzymes associated with elements of the Golgi complex.

The early, high-mannose sugar structures are susceptible to the activity of endoglycosidase H, an enzyme which specifically cleaves these oligosaccharides from their associated polypeptides (Kobata, 1979). Arrival of polypeptides at the cis cisterna of the Golgi complex is generally heralded by the action of two α-mannosidase activities specifically localized to this compartment which function in a pair of tandem reactions to trim five of the mannose residues from the sugar structures of most newly synthesized proteins. The loss of these mannose residues is accompanied by a loss of sensitivity to endoglycosidase H (Kornfeld and Kornfeld, 1985). Consequently, endoglycosidase H has proved extremely useful in studies on the biosynthesis of membrane proteins by serving as a biochemical marker for the progression of newly synthesized polypeptides from the ER through the early Golgi.

Once they have been divested of six mannose residues, the newly shorn oligosaccharides are remodeled by enzymes associated with the medial and trans elements of the Golgi stack. Transport of proteins among Golgi stacks is accomplished by vesicles bearing a non-clathrin coat, which is presumably identical to that associated with the vesicles which mediate ER to Golgi traffic (Orci et al., 1986). Sugar transferases add N-acetylglucosamine, galactose, and sialic acid residues in strict series, with the action of each transferase being rigorously dependent on the biosynthetic history of the oligosaccharide structure up to that point (Kornfeld and Kornfeld, 1985). While all N-linked sugars begin with the same dolichol intermediate, the final complex oligosaccharide produced by the actions of the Golgi enzymes varies from protein to protein. Apparently, different proteins have different affinities for or susceptibilities to the individual subsets of lumenally disposed sugar transferases which they encounter as they transit the Golgi complex. Since each stage of the modification is in part determined by its predecessor, small variations in the degree of processing can produce large compositional and structural alterations in the final constellation of sugars sculpted by the Golgi processing enzymes. Once again, the function of these sugars and the role that this compositional variation plays in dictating the various properties of membrane proteins remain largely unknown.

2. Other Covalent Modifications

The Golgi complex contributes a number of other posttranslational modifications to newly synthesized membrane proteins. The addition and maturation of O-linked sugars (which are attached to the hydroxyl groups of serine and threonine residues) are thought to occur entirely within the confines of the Golgi stacks (Hanover *et al.*, 1982; Cummings *et al.*, 1983). Similarly, phosphorylation and sulfation of sugars, proteins, and proteoglycans have been conclusively associated with the Golgi apparatus (Kornfeld and Kornfeld, 1985; Huttner, 1988). While it is beyond the scope of this review to discuss the mechanisms of each of these covalent modifications in detail, it is important to point out that, as with the processing of N-linked sugars, these reactions are thought to occur along a temporal and spatial assembly line. The enzymes responsible for the individual steps in the modification are compartmentalized, that is, restricted in their distribution to only a subset of the Golgi cisternae. Thus, the action of each enzyme occurs in a spatial and temporal sequence. At each station in its saltatory progress through the Golgi stacks the newly synthesized protein encounters one component of the processing machinery at a time, in isolation from the other enzymes of this system.

3. Stabilization of Conformation

Recent evidence from a number of laboratories suggests that the modifications proteins experience while transiting the Golgi complex are not limited to the covalent variety discussed above. Studies on the trimeric hemagglutinin (HA) protein of the influenza virus indicate that it undergoes a "stabilization" of its tertiary structure during or soon after its residence in the Golgi. Copeland *et al.* (1986) found that influenza HA protein forms its characteristic homotrimers while still in the membranes of the RER. Biochemical analysis, however, reveals that these early trimers are not bound together as tightly as those which populate the cell surface membrane. Solubilization of the newly synthesized trimers under fairly gentle detergent conditions can lead to the dissolution of the complex, whereas the mature plasmalemmal trimers are resistant to this treatment. These investigators found that the transition from the unstable to the stable conformer occurs while the protein is passing through or out of the Golgi complex. The nature of the modification (if any) which brings about this stabilization remains unknown. It is interesting to note, however, that Skibbens *et al.* (1989) have found that an intramolecular disulfide bond in the HA protein is lost at roughly the same point in this protein's posttranslational processing that stabilization is achieved. It seems likely, therefore, that during its time in the Golgi (or not long thereafter) the protein modifies

its folding pattern in a manner which affects both its own tertiary structure and its affinity for oligomerization.

E. The Role of the Golgi Complex: The Trans Golgi Network and Delivery to the Cell Surface

1. The Functions of the Trans Golgi Network

For most plasma membrane proteins, the staging area for delivery to the cell surface is the final cisterna of the Golgi apparatus, which has come to be referred to as the trans Golgi network, or TGN. The TGN is morphologically discernable from the other Golgi stacks and appears to be endowed with a number of properties not shared by other Golgi elements (Griffiths and Simons, 1986). For example, addition of sialic acid appears to occur entirely within the confines of the TGN (Fuller *et al.*, 1985). Furthermore, newly synthesized proteins accumulate within the TGN when their progress to the cell surface is impeded by lowering the temperature to 20°C (Matlin and Simons, 1983; Griffiths *et al.*, 1985; Saraste and Kuismanen, 1984). Most importantly for the purposes of this review, the TGN appears to be the site at which proteins bound for different subcellular compartments become segregated from one another. Biochemical and immunocytochemical experiments performed on a number of systems have revealed that polypeptides destined for different subcellular locales remain together as far as the TGN and become separated from one another within its confines (Fuller *et al.*, 1985; Rindler *et al.*, 1984; Orci *et al.*, 1987; Tooze *et al.*, 1987). This sorting function of the TGN is discussed at greater length in the next section of this review.

Proteins depart the TGN in clathrin-coated vesicles, which tend to bud from the lateral boundaries of this structure's membranous stack (Orci *et al.*, 1984). Vesicles emanating from the TGN carry newly synthesized polypeptides to a large number of cellular destinations, including prelysosomal endosomes (Griffiths *et al.*, 1988), secretory granules, and the cell surface. As has been discussed above, the trafficking of these vesicles to their appropriate subcellular targets is likely to be dependent on cytosolic factors such as NSF and SNAPs [soluble NSF attachment proteins, which appear to link NSF to the membranes of vesicular carriers (Clary *et al.*, 1990)] as well as GTP and small ras-like GTP-binding proteins. It has been shown in yeast, for example, that fusion of secretory granules with the cell surface is prevented by mutations in a ras-like GTP-binding protein encoded by the *Sec 4* gene (Salminen and Novick, 1987).

2. Cell Surface Delivery

After departing the TGN, the secreted products of cells are frequently packaged into secretory granules which are stored in the cytoplasm until some physiological stimulus initiates their fusion with the plasmalemma and the discharge of their content (Burgess and Kelly, 1987). In contrast, no such regulated delivery pathway exists for most plasmalemmal poly-peptides. The vast majority of plasma membrane proteins are delivered directly and constitutively from the TGN to the cell surface. They are not accumulated in any storage compartment within the cell and their arrival at the cell surface is not triggered by a physiological stimulus.

There are, however, at least four exceptions to this general rule. The gastric H^+/K^+-ATPase (Urushidani and Forte, 1987), the H^+-ATPase of renal intercalated cells (Schwartz and Al-Awqati, 1986), the insulin-sensitive glucose transporter of adipocytes (Blok *et al.*, 1988), and the water channels of the renal collecting duct (Handler, 1988) are all resident in cytosolic vesicles whose fusion with the cell surface membrane is under rigid physiological control. Thus, stimulation of gastric acid secretion by histamine is associated with the insertion of an enormous intracellular pool of H^+/K^+-ATPase into the gastric parietal cell's apical plasma membrane (Urushidani and Forte, 1987). Similarly, ADH induces the fusion of water channel-containing vesicles with the apical membrane of collecting duct epithelial cells (Handler, 1988), and insulin initiates the delivery of intra-cellular stores of glucose channels into the adipocyte plasma membrane (Blok *et al.*, 1988). In this manner, the cells are able to carefully and rapidly influence the activity of membrane transport processes without directly modulating the catalytic activities of the transporters themselves. This adaptation may have evolved to control the function of transport systems whose structural characteristics render them, for some reason, poor candi-dates for regulation by covalent or allosteric modification.

Removal of the stimulus for membrane insertion results in the retrieval of these transport proteins from the cell surface via an endocytic process. This endocytosis does not, however, appear to involve traditional acidic endosomes and lysosomes (Lencer *et al.*, 1990). Instead, the recycled proteins appear to be returned to their storage vesicles or, alternatively, those storage vesicles are regenerated through the endocytic process. The mechanisms through which these "stored" membrane proteins are tar-geted from the TGN (and subsequently the cell surface) to their intracellu-lar pools remain to be established. Furthermore, the generality of this pathway—its applicability to other transport proteins or to membrane proteins in general in other cell types—is not at all clear. This specializa-tion is, however, an excellent example of the cellular capacity discussed in

the Introduction, that is, the ability to control temporally and spatially the composition of the plasma membrane.

III. SORTING AND EPITHELIAL POLARITY

In Section II of this review, a general outline of the postsynthetic pathway pursued by newly synthesized plasma membrane proteins was presented. The generic pathway described above is more or less applicable to any plasmalemmal polypeptide in any animal cell type. It can be fairly well encompassed by the observation that a newly synthesized membrane protein's progress to the cell surface is marked by its saltatory passage through distinct subcellular processing stations (see Fig. 1). While essentially correct, however, this summation does not appropriately emphasize a critically important theme which is common to each step in this pathway. During each phase in a membrane protein's processing, it must be examined by some mechanism capable of determining whether the organelle it currently occupies is in fact its site of ultimate functional residence. Those proteins which are identified by this mechanism as belonging to the organelle in which they reside must be retained, while those deemed inappropriate must be allowed to travel to the next processing locus. In other words, the RER must be able to hold on to newly synthesized components of the RER membrane while allowing proteins destined for the Golgi and the cell surface to proceed. Similarly, each subcompartment of the Golgi must be able to recognize and retain its constituent proteins from among the traffic of newly synthesized membrane proteins which passes through them.

The nature of this mechanism is beginning to be elucidated. Sequences have been identified on the carboxy termini of resident ER proteins which mediate their retention in the ER (Nilsson *et al.*, 1989). A sequence in the transmembrane domain of the Golgi protein has been shown to be important in its Golgi localization (Machamer and Rose, 1987). Clearly, some primary, secondary, or tertiary structural determinants of membrane proteins must contain the information that is used by the cell to target them to the appropriate destination. Furthermore, the cell must possess some machinery which is capable of interpreting and acting on these signals. Every newly synthesized membrane protein which enters the cell's endomembranous processing network must be sorted, actively or passively, and targeted to the correct subcellular destination.

The sorting problem is compounded in polarized epithelial cells. Epithelial cells line body cavities, essentially forming the barrier between the inside and the outside of an organism. It is the selective permeability
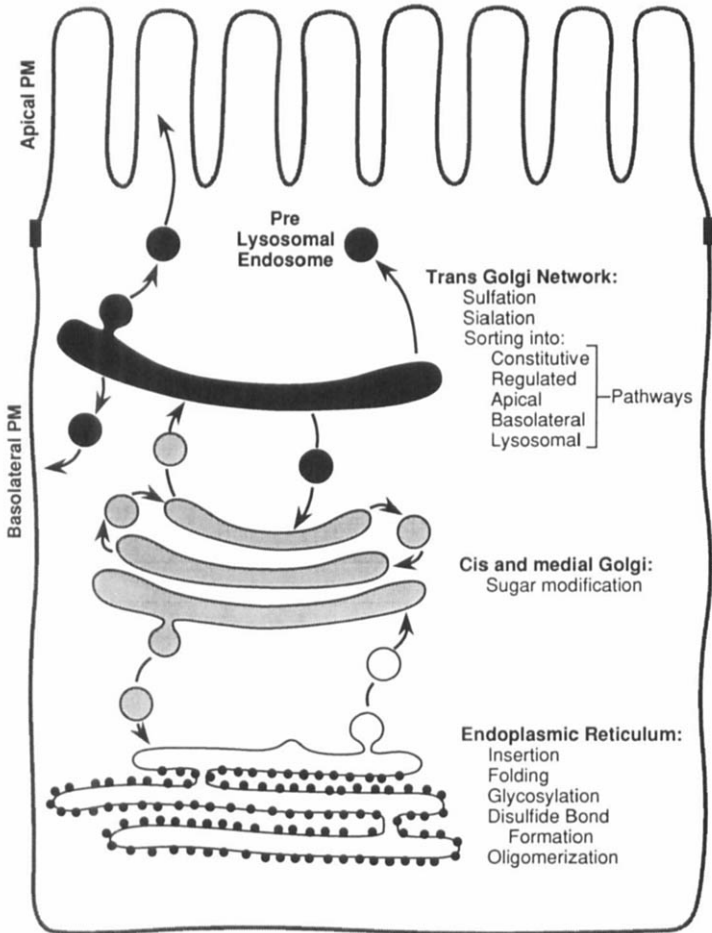
FIG. 1. Newly synthesized plasma membrane (PM) proteins undergo a wide variety of modifications and sorting operations en route to the cell surface. As described in the text, each stage in the postsynthetic maturation of a plasmalemmal protein is associated with unique processing functions. Each stage is also endowed with the capacity to both determine a protein's subsequent destination and mediate its appropriate targeting. In this sketch, drawn to represent a typical epithelial cell, notable events in a plasma membrane protein's postsynthetic course are indicated next to the organelles in which they occur. See text for details.

barriers provided by epithelia which control the exchange of solutes and fluid between an organism and its environment. The plasma membranes of polarized epithelial cells are divided into two morphologically and biochemically distinct domains (Simons and Fuller, 1985; Matlin, 1986; Caplan and Matlin, 1989; Rodriguez-Boulan and Nelson, 1989). The apical membrane, which is frequently endowed with microvilli, generally faces the lumen of a body compartment which is topologically continuous with the extracorporeal space. The basolateral plasmalemma rests on the epithelium's basement membrane and is in contact with the extracellular fluid space. Both the phospholipid and polypeptide compositions of the two domains are markedly different. The boundary between the apical and basolateral cell surfaces is delineated by tight junctions, which impede the passage of molecules between the two extracellular fluid compartments (see Fig. 2).

Perhaps the most important physiological feature of epithelial cells is their capacity to carry out vectorial transport, that is, the net movement of substances from one extracellular fluid compartment to the other, frequently in the face of steep unfavorable concentration gradients. The ability of transporting epithelia to mediate vectorial transport is explained by the asymmetric distribution of transport proteins among their two plasmalemmal surfaces (Schultz, 1986). Movement of a substance from one compartment to the other requires the participation of both apical and basolateral transporters working in series. The differential placement of pumps, cotransporters, and channels in the apical and basolateral plasma membranes bestows on epithelia their ability to carry out unidirectional and uphill transport. The value of this asymmetry is nicely illustrated by the principal cell of the renal collecting duct (O'Neil, 1987; Koeppen and Giebisch, 1985). The basolateral plasma membrane of the principal cell is extremely rich in Na^+/K^+-ATPase. This complement of sodium pump functions to keep the cytosolic concentration of sodium low. A sodium channel in the apical membrane allows sodium to flow down its concentration gradient from the tubule lumen into the cytoplasm. Apically entering sodium is expelled from the cytoplasm via the basolateral Na^+/K^+-ATPase and is thus transported from the lumenal to the extracellular fluid compartment. The energy of the sodium gradient generated by the Na^+/K^+-ATPase is exploited to drive the energetically unfavorable resorption of sodium from the renal tubule. This scheme is critically dependent on its geometry. It only works if the sodium pump and the sodium channel are present in different membrane surfaces which are separated by relatively sodium-impermeable tight junctions. Clearly, the cell must possess some mechanisms which are capable of organizing and maintaining this geometry.
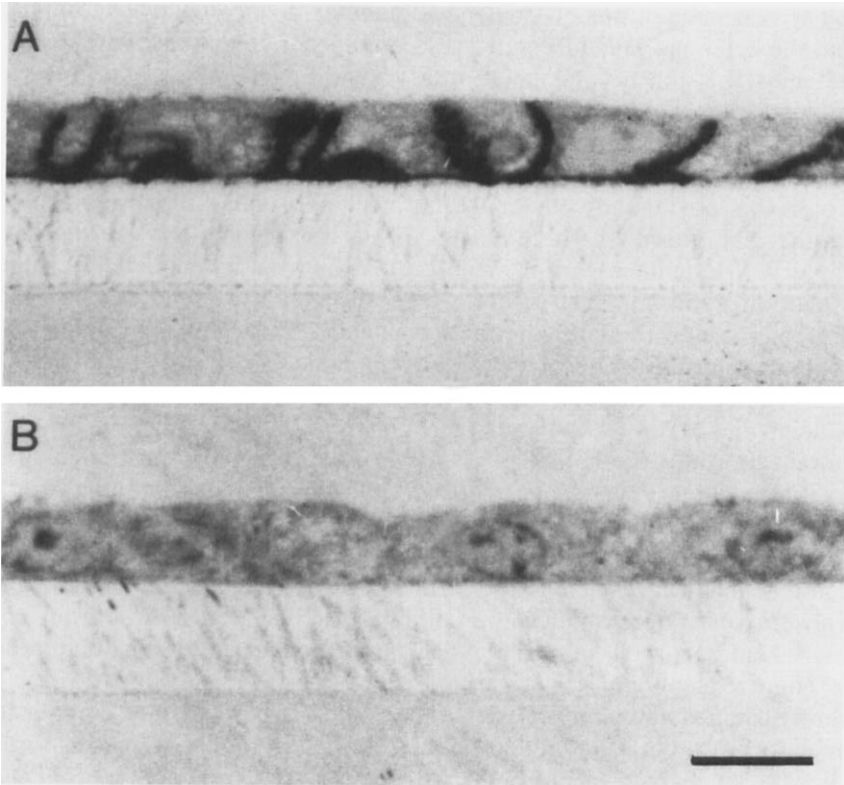
FIG. 2. The plasma membranes of polarized epithelial cells are divided into two biochemically distinct domains. The protein compositions of the apical and basolateral membranes of an epithelial cell are essentially distinct. In A, cultured canine kidney epithelial cells (MDCK) grown on permeable filter supports were labeled for immunoelectron microscopy with an antibody directed against the Na^+/K^+-ATPase α-subunit followed by a secondary reagent coupled to horseradish peroxidase. Dense reaction product is associated with only the lateral and basal membranes. No sodium pump can be detected on the apical surface. No staining is detected when a non-immune IgG is employed (B). Bar, 10 μm. (From Caplan *et al.*, 1986.)

Asymmetric distributions are not limited to the epithelial polypeptides which participate in ion transport. The vast majority of plasmalemmal proteins in polarized epithelial cells are restricted in their distributions to one or the other cell surface domain. For the purposes of this discussion, therefore, it is useful to think of an epithelial cell's plasma membrane as two distinct organelles which, although physically contiguous, are equipped for specific and unique functions.

   The existence of this plasmalemmal compositional anisotrophy implies
that the cell possesses mechanisms to both generate and maintain it. The
cell must be able to recognize newly synthesized membrane proteins
bound for one or the other cell surface domain, target them to their
appropriate destination, and retain them there following their arrival. As
was discussed in the context of the resident proteins of the ER and the
Golgi, this postulated cellular sorting capability must, in some form or
another, be based on the concepts of sorting signals and sorting ma-
chinery. We define a sorting signal as that information encoded in some
aspect of a protein's structure which the cell uses to determine the
protein's site of ultimate functional residence. Sorting machinery, in this
context, refers to all of the cellular components involved in recognizing,
interpreting, and acting on the information contained in sorting signals
(Caplan and Matlin, 1989).

   Research into the nature of epithelial sorting signals and the cellular
sorting machinery is now over a decade old. A tremendous amount of work
in this field was inspired by a seminal observation reported in 1978 by
Rodriguez-Boulan and Sabatini, which made it clear that this problem
could be made accessible to the techniques of cell and molecular biology.
These investigators studied the budding of enveloped viruses from po-
larized epithelial cells in culture. They took advantage of the fact that a line
of polarized epithelial cells derived from the dog renal tubule (Madin–
Darby canine kidney, or MDCK; Madin and Darby, 1975) is susceptible to
infection with both the influenza virus and the vesicular stomatitis virus
(VSV). Through an electon microscopic analysis, they noticed that the
influenza virus buds predominantly from the apical surface of infected
cells, while VSV buds predominantly from the basolateral plasmalemma.

   The encapsulating lipid bilayer membranes of enveloped viruses are
notable for an extremely high density of transmembrane "spike" glyco-
proteins. In immunoelectron microscopic studies, Rodriguez-Boulan and
Sabatini found that, prior to viral budding, the viral spike glycoproteins
synthesized by infected cells accumulate in the plasma membrane domain
from which budding will occur. Thus, influenza HA protein is targeted to
the apical plasma membrane, where it awaits incorporation into virions.
Similarly, the VSV G protein behaves as a basolateral plasma membrane
protein until it becomes associated with the departing viral envelope. This
observation was immensely important in that it provided investigators in
the field of epithelial polarity with the first model system for membrane
protein sorting whose components could be readily manipulated to suit an
experimental design.

   Within a few years after these initial observations, the genes encoding

the influenza HA and VSV G proteins were cloned and expressed by transfection in MDCK cells. In a number of studies it was found that the spike glycoproteins synthesized by transfected cells still accumulate on their characteristic membrane domains, even in the absence of all of the other viral components that are normally synthesized during the course of infection (Stephens *et al.,* 1986; Gottlieb *et al.,* 1986b; Roth *et al.,* 1983; Jones *et al.,* 1985). For all intents and purposes, it could be said that the influenza HA protein behaves like a bona fide apical protein and the VSV G protein like a bona fide component of the basolateral plasmalemma. From these experiments it was clear that all of the information necessary to target these polypeptides to their respective cell surface domains resides within the proteins themselves. Their sorting is not dependent on any contribution from the viral genome. These studies comprised the first direct demonstrations that epithelial sorting signals exist and are wholly determined by the characteristics of the sorted proteins themselves.

Over the ensuing years, a tremendous amount of effort has been devoted to uncovering the nature of these signals and of the sorting machinery which interprets them. At this point in time, it is safe to say that neither of these elemental components of the epithelial sorting system is well understood. Identifying putative sorting signals and components of the cellular sorting machinery has proved extremely difficult for both technical and theoretical reasons. While the mechanisms underlying sorting have yet to be elucidated, however, a tremendous amount has been learned which offers clues into the nature of the sorting process. A synopsis of this literature is presented below, divided somewhat arbitrarily into discussions of sorting pathways, sorting signals, sorting machinery, and the biogenesis and maintenance of the polarized state.

A. Sorting Pathways

The first, and perhaps most accessible, question explored in the field of epithelial polarity relates to where, within the cell, sorting occurs. Three experimentally distinguishable alternatives were proposed to describe the route taken by newly synthesized plasmalemmal polypeptides on their way to the cell surface (Evans, 1980). These models can be identified as vectorial sorting, random sorting, and obligate missorting. The vectorial sorting paradigm predicts that all of the operations required to target a protein to the appropriate cell surface domain occur prior to that protein's arrival at the cell surface. According to this scheme, sorting is an intracellular process and transport of newly synthesized plasmalemmal proteins to

the cell surface is vectorial in the sense that a polypeptide's first appearance at the cell surface is coincident with its arrival at the membrane domain in which it rightly belongs.

According to the random sorting model, no sorting occurs prior to cell surface delivery. Apical and basolateral proteins depart the TGN together and are delivered without preference to both cell surface domains. In this formulation, sorting is the product of selective endocytosis, which removes misplaced proteins from the cell surface and reroutes them to the appropriate domain. Obligate missorting is something of a compromise between the mutally exclusive vectorial and random proposals. This model predicts that apically and basolaterally directed proteins depart the TGN together, perhaps in the same vesicular carrier, and are delivered together to one of the two plasmalemmal surfaces. The subset of proteins for which this delivery process is correct, i.e., those which arrive directly at their proper destination, are allowed to remain in place. The polypeptides which find themselves in the incorrect membrane domain as a result of the initial delivery step are retrieved by endocytosis and shuttled transcytotically to the opposite surface. In theory, the obligate misdelivery model is equally compatible with the initial membrane insertion step occurring at either the apical or the basolateral pole of the cell. In practice, evidence has been gathered supporting only the latter possibility.

The first studies of sorting pathways were performed on MDCK cells infected with either the influenza virus or VSV. Cells were metabolically labeled with [^{35}S]methionine and exposed at their apical or basolateral surfaces to either trypsin or to antibodies directed against the ectodomains of the viral spike glycoproteins (Misek et al., 1984; Matlin and Simons, 1984; Pfeiffer et al., 1985). The susceptibility of these proteins to proteolysis or to antibody binding was assessed by immunoprecipitation followed by gel electrophoresis and fluorography. It was found that the VSV G protein was never even transiently available for interaction with apically added antibody or vulnerable to the action of apically added trypsin (Pfeiffer et al., 1985). Similarly, the influenza HA protein was only affected by these treatments when they were applied from the apical side (Misek et al., 1984; Matlin and Simons, 1984). These findings were most consistent with the vectorial sorting model, which would predict that the newly synthesized spike glycoproteins would appear first and only at their appropriate membrane surfaces and would thus never be available to reagents added at the opposite side.

Subsequent studies have since verified that, in MDCK cells, vectorial sorting applies to endogenous proteins as well. The pathway followed by
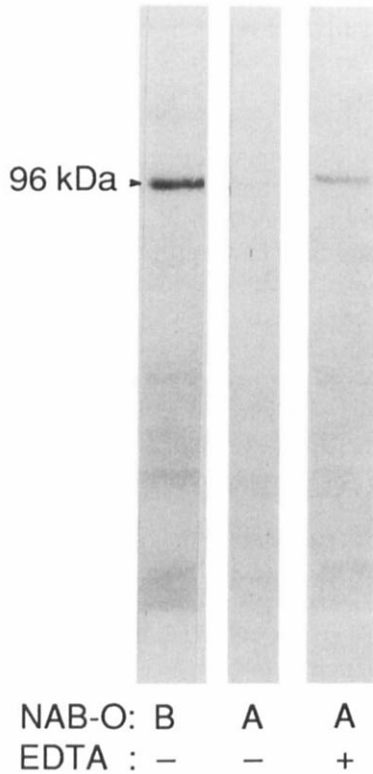
FIG. 3. The newly synthesized sodium pump is sorted vectorially to the basolateral plasma membrane in MDCK cells. MDCK cells were grown on a filter, as depicted in Fig. 2. Following pulse labeling with [^{35}S]methionine, they were exposed to NAB-ouabain at their apical or basolateral surfaces, photolyzed, solubilized, and subjected to immunoprecipitation with anti-ouabain antibodies as described in the text. As can be seen in the figure, NAB-ouabain had access to the newly synthesized Na$^+$/K$^+$-ATPase when it was added to the basolateral (B) but not the apical (A) medium compartment. Rupturing intercellular tight junctions through calcium chelation allowed apically added NAB-ouabain to interact with the newly synthesized sodium pump (EDTA +). These experiments indicate that the sodium pump does not appear, even briefly, on the apical surface prior to its arrival at the basolateral plasmalemma. (From Caplan et al., 1986.)

the newly synthesized Na$^+$/K$^+$-ATPase was assessed using a protocol similar to that described above in the experiments relating to intracellular activation of the sodium pump (Caplan et al., 1986, 1990) (see Fig. 3). MDCK cells were grown on permeable filter supports in order to allow for simultaneous and independent access to both membrane surface domains. Following a brief pulse labeling with [^{35}S]methionine, the photo-

activatable NAB derivative of ouabain was added to the apical or baso-
lateral medium compartment. After a 90-min chase incubation in the
presence of this photoaffinity reagent, bound NAB-ouabain was cova-
lently incorporated into the Na^+/K^+-ATPase α-subunit through exposure
to UV light. Membranes prepared from these cells were subjected to
immunoprecipitation with an anti-ouabain antibody and immunoprecipi-
tates were analyzed by gel electrophoresis followed by fluorography. If
newly synthesized sodium pump appeared, even briefly, on the apical
surface, then radiolabeled α-subunit would have been present in immuno-
precipitates from cells exposed to apical NAB-ouabain during the chase.
Analysis of the fluorographs, however, revealed that newly synthesized
Na^+/K^+-ATPase was only accessible to NAB-ouabain when this com-
pound was added to the basolateral medium. It was possible to conclude
from these experiments that less than 5% of the newly synthesized so-
dium pump appeared, stably or transiently, in the apical plasma mem-
brane.

Similar experiments examining the sorting of endogenous MDCK
proteins have taken advantage of biotin coupled to the amine-reactive
NHS group to label selectively proteins expressed at the apical and baso-
lateral surfaces of filter grown cells (Lisanti *et al.*, 1989b; Le Bivic *et al.*,
1990). Metabolically labeled proteins accessible to the biotin probe were
isolated on avidin-conjugated Sephrarose beads and analyzed by gel elec-
trophoresis followed by fluorography. Proteins which are normally resi-
dent in the basolateral plasma membrane were never accessible to apically
added NHS-biotin throughout the course of their posttranslational
processing. Apical proteins were similarly invulnerable to conjugation
with basolaterally applied reagent. Taken together, all of these results
provide a strong demonstration that sorting in MDCK cells occurs intra-
cellularly and is complete prior to the arrival of newly synthesized mem-
brane proteins at the cell surface.

The observation that MDCK cell sorting is vectorial invites curiosity
about the intracellular site at which sorting occurs. Rindler *et al.*, exam-
ined this question by performing immunoelectron microscopy on MDCK
cells which had been doubly infected with both the influenza virus and
VSV (Rindler *et al.*, 1984). These investigators found that the influenza
HA and VSV G proteins could be colocalized in all of the intracellular
membranous structures involved in membrane protein processing up
through the TGN. Segregation of the apical from the basolateral proteins
seemed to happen at or beyond this point.

Similar observations were made in a biochemical investigation per-
formed by Fuller *et al.* (1985). Their experiment took advantage of the fact
that, in addition to the HA protein, the influenza viral genome encodes a

transmembrane neuraminidase which is incorporated into the apical plasma membrane and subsequently into viral envelopes. As was mentioned in the previous section, sialic acid is added in the TGN (Fuller *et al.*, 1985; Griffiths and Simons, 1986). Furthermore, it must be noted that incubation of cells at 20°C allows newly synthesized proteins to progress only as far as the TGN (Matlin and Simons, 1983; Saraste and Kuismanen, 1984; Griffiths *et al.*, 1985). Warming the cells to 37°C relieves the temperature block and allows the accumulated polypeptides to exit the TGN and proceed to the cell surface. Fuller *et al.*, found that when VSV-infected cells were incubated at 20°C, the G protein, which is normally multiply sialated, became hypersialated. This is not surprising, since the 20°C block traps the G protein in the compartment containing the sialyl transferase. Interestingly, however, when MDCK cells doubly infected with VSV and influenza virus were incubated at 20°C, the G protein was found to be markedly hyposialated. This observation was taken as an indication that the VSV G protein and the influenza neuraminidase had physical access to one another during their temperature-induced confinement in the TGN. This conclusion fit well with Rindler *et al.*'s (1984) morphological assessment that apically and basolaterally directed proteins were still intermingled at the TGN stage of their processing.

Immunoelectron microscopic studies have also been performed on nonpolarized endocrine cells which manifest regulated and constitutive secretory pathways. It was noted that, in the TGN, proteins destined for packaging into regulated secretory granules were physically segregated from membrane and secretory proteins as well as from intraluminal viral particles, all of which depart the Golgi for the cell surface via the constitutive route (Orci *et al.*, 1987; Tooze *et al.*, 1987). Taken together with the results discussed above, these observations suggest that sorting begins in the TGN and is completed by the time (or certainly not long after) newly synthesized polypeptides depart this final recognizable locus of the intracellular processing pathway.

While the vectorial model almost certainly applies to MDCK cells, it must be noted that other epithelial cell types appear to target membrane proteins to their cell surfaces via a different sorting pathway. In cell fractionation studies, Bartles *et al.*, (1987) followed the postsynthetic route pursued by two apical proteins in hepatocytes. They concluded that both proteins appeared in a fraction derived principally from the basolateral plasma membrane prior to being detectable in membranes cosedimenting with the apical plasmalemma. This observation suggested that these polypeptides might be targeted from the Golgi first to the basolateral surface and subsequently transcytosed to the apical side. This mode of cell surface delivery would conform to the obligate missorting model presented

above. Interestingly, this route also mirrors the course followed by the polymeric immunoglobulin receptor of hepatocytes (Geuze *et al.*, 1984; Hoppe *et al.*, 1985). This transmembrane protein is delivered from the Golgi to the basolateral cell surface, where it binds IgA circulating in the blood. The IgA–receptor complex is endocytosed and transported to the apical membrane, at which point the receptor's ectodomain is cleaved from its transmembrane anchor. The ectodomain fragment, referred to as secretory component, is released into the bile in association with the bound IgA.

Cell fractionation-based and cell surface labeling studies of the sorting pathways in intestinal cells have also provided evidence for some manner of obligate missorting pathway. Metabolic pulse labeling and cell fractionation were used to follow the brush border enzymes aminopeptidase N (Massey *et al.*, 1987) and sucrase-isomaltase (Hauri *et al.*, 1979) in small intestinal enterocytes. Analysis of immunoprecipitates from the various membrane fractions suggested that this protein complex was associated with the basolateral plasma membrane prior to arriving at the apical surface. While all of these results are extremely interesting, cell fractionation studies are always open to the criticism that difficult-to-control-for and difficult-to-measure cross-contamination may artifactually skew the results. Similar experiments in other laboratories suggest that microvillar proteins are vectorially sorted in intestinal cells (Danielsen and Cowell, 1985).

More recent experiments have taken advantage of the biotin labeling protocol outlined above to observe the behavior of plasma membrane proteins synthesized by a polarized human intestinal adenocarcinoma cell line SK-CO-15 (Le Bivic *et al.*, 1989) and by the intestinal carcinoma cell line Caco-2 (Matter *et al.*, 1990). These experiments revealed that, while basolateral proteins were targeted vectorially in both cell systems, the behavior of apical proteins was somewhat more complicated. Apical proteins were vectorially targeted in the SK-CO-15 cells, whereas in the Caco-2 cells only a portion of the newly synthesized apical proteins were vectorially sorted and made their initial plasmalemmal appearance at the apical cell surface. The remainder were initially accessible to basolaterally added NHS-biotin and were subsequently transcytosed to the apical plasma membrane. Apparently, these cells handle apical plasmalemmal proteins via some combination of the vectorial and obligate missorting pathways.

It is perhaps somewhat surprising that the sorting pathway, which one might be tempted to regard as a fundamental property of all epithelial cells, appears to vary from one cell type to another. A possibile explanation for this puzzling variability, however, might be found in the tissue-specific

secretory behavior manifest by epithelial cells. It has been demonstrated that MDCK cells release secretory proteins into both the apical and basolateral media compartments. (Kondor-Koch et al., 1985; Gottlieb et al., 1986a; Caplan et al., 1987). Furthermore, it has been shown that the default pathway for secretory proteins, that is, the route pursued by secretory proteins incapable of interacting with the cellular sorting machinery, is apical and basolateral (Kondor-Koch et al., 1985; Gottlieb et al., 1986a; Caplan et al., 1987). The default pathway is thought to reflect to some extent the relative volume, or capacity, of apically and basolaterally directed membrane carriers which are available to bulk flow cargo.

In contrast to the example of MDCK cells, hepatocytes seem to have no direct secretory pathway to the apical surface. While a huge volume of secretory proteins is released at the basolateral membrane into the hepatic sinusoids, no proteins have been identified which are secreted directly at the apical surface into the bile canaliculi. Furthermore, studies on the default pathway associated with Caco-2 cells indicated that the bulk of unsorted polypeptides are released basolaterally by this cell line (Hughson et al., 1989; Rindler and Traber, 1988). Taken together, these observations suggest that the membrane protein sorting model associated with a given cell type may reflect the relative activities of its apical and basolateral secretory pathways. In hepatocytes, for example, the absence of an apical secretory pathway might reflect, or be responsible for, an absence of membranous traffic from the Golgi to the apical cell surface. Thus, apically bound proteins in hepatocytes may have no choice but to depart the TGN in basolaterally bound vesicles. The same could be said for Caco-2 cells, in which the basolaterally directed default pathway may reflect a paucity of apically directed vesicular carriers.

This model is currently favored, since it seems to correlate two separate cellular behaviors. It also raises a number of interesting and difficult to answer questions. For example, is the existence of an apically directed secretory cargo required in order to maintain vesicular traffic from the TGN to the apical surface? Conversely, is there no direct apical secretory cargo in certain epithelial cell types precisely because there is no direct route to the apical surface? In those cells which employ obligate misdelivery of apical proteins to the basolateral surface, do the apical and basolateral proteins occupy the same vesicular carriers, or are they sorted from one another in the TGN and carried to the basolateral surface in separate, segregated shuttles? What is the endocytic sorting mechanism that allows these cells to recognize apical proteins in the basolateral surface and remove them for transort to their appropriate destination? These questions, although somewhat arcane, are of much more than academic interest. Their solutions, if obtainable, will tell us a tremendous amount

about the control of intracellular membrane traffic and the mechanisms of membrane protein targeting.

B. Sorting Signals

As was mentioned above, the demonstration that the VSV G and influenza HA proteins, expressed by transfection in MDCK cells, were sorted with high fidelity to the appropriate cell surface domains provided the first solid evidence that sorting signals are wholly contained within some aspect of the structure of the sorted molecule itself. Experiments by a number of groups had also shown that blocking the addition of N-linked sugars to the viral spike glycoproteins [either through the action of the glycosylatin inhibitor tunicamycin (Green *et al.*, 1981; Roth *et al.*, 1979) or by infecting strains of MDCK cells defective in glycosylation (Green *et al.*, 1981)] had no effect on the sorting of these polypeptides. In light of these observations, it seems certain that the information required to specify sorting is encoded in the amino acid sequence of a polypeptide, either directly or by virtue of the tertiary structure this sequence effects. A large body of literature has now developed out of the attempts to identify, or at least narrow, the search for these sorting signals. As of this writing, it is safe to say that nothing even remotely approximating an answer is yet available, although a few general rules may be emerging. The most popular approaches in the hunt for sorting signals have involved the construction of chimeric or truncated versions of the viral spike glycoproteins, whose sorting could be analyzed in transfected cells. These experiments have produced very complex and frequently contradictory results. Since this field is rather tangled and has been reviewed fairly recently (Caplan and Matlin, 1989), it is simply summarized here.

Truncated forms of the influenza HA protein which lack cytosolic and transmembrane domains are secreted from transfected epithelial cells. Analysis of this secretion reveals that it is predominantly apical (Roth *et al.*, 1987). It should be noted, however, that another group has found that a similar construct may be released into both the apical and basolateral compartments (Gonzalez *et al.*, 1987). Similar anchor-minus constructs of the VSV G protein (and of the basolaterally directed spike glycoprotein of the Friend mink leukemia virus) are released from MDCK cells into both the apical and basolateral media (Gonzalez *et al.*, 1987; Stephens and Compans, 1986). Chimeras composed of the influenza HA ectodomain and the VSV G transmembrane and endodomains are sorted apically (Roth *et al.*, 1987; McQueen *et al.*, 1986). The complementary constructs, bearing

the VSV G ectodomain wedded to the influenza HA transmembrane and endodomains, are probably basolaterally targeted (McQueen *et al.*, 1987; Puddington, *et al.*, 1987; Compton *et al.*, 1989). These results seem to suggest that the ectodomains are important for the sorting of both apical and basolateral proteins, although the random secretion of the anchor-minus VSV G truncation is not entirely consistent with this formulation.

Recent studies have demonstrated that proteins anchored to the membrane via glycophosopholipids are expressed by epithelial cells essentially exclusively in the apical plasmalemma (Lisanti *et al.*, 1988, 1990). The members of this fascinating class of membrane proteins, which includes within its roster alkaline phosphatase, 5'-nucleotidase, and trypanosomal surface antigens, are intially synthesized on bound polysomes as trans-membrane proteins (Cross, 1987). While still in the membrane of the RER they are cleaved from their transmembrane portion and transferred cova-lently to a lumenally facing glycosyl-phosphatidylinositol molecule (GPI). Two groups have tested the possibility that attachment to a GPI anchor is in itself a sufficient signal to ensure apical targeting. Brown *et al.* (1989) engineered a construct in which the VSV G ectodomain was joined with the lipid acceptor site of Thy-1, a GPI-coupled lymphocyte antigen. When expressed in MDCK cells, the resultant lipid-linked G protein was sorted to the apical plasmalemma. When placental alkaline phosphatase (PLAP) was expressed in MDCK cells, this GPI-linked protein was delivered to the apical membrane. An anchor-minus form of PLAP, which lacks the lipid association, was secreted both apically and basolaterally in a roughly 2 : 1 ratio. Attachment of PLAP to the VSV G transmembrane domain and cytosolic tail resulted in a chimeric protein that was targeted basolaterally. Similar results were gathered by Lisanti *et al.* (1989a). These investigators coupled the GPI-linked tail of decay-accelerating factor (DAF) to the ectodomain of herpes simplex glycoprotein D (a basolateral protein) and to human growth hormone (normally targeted for regulated secretion). Both constructs were delivered to the apical surface of transfected MDCK cells. These observations seem to support the notion that lipid attachment might serve to convey an apical sorting signal; however, it is difficult to synthe-size all of these experiments into a single coherent picture.

A cDNA encoding the polymeric immunoglobulin receptor has been transfected into MDCK cells. Remarkably, this protein retraces in these cultured kidney cells the complicted course it follows in hepatocytes (Mostov and Deitcher, 1986). The receptor first appears at the basolateral surface, from which it is endocytosed and transported to the apical mem-brane. Immediately before or after its arrival at the apical surface the receptor undergoes a proteolytic cleavage which releases the secretory component into the apical media. The fact that this protein follows its

rather indirect course in a cell type which normally manifests vectorial sorting has prompted the suggestion that the polymeric immunoglobulin receptor might possess hierarchical sorting signals, that is, two sorting signals whose expression is temporally or spatially determined. According to this line of thought, a basolateral sorting signal predominates during the protein's initial voyage from the TGN to the basolateral cell surface. After basolateral delivery, however, an apical signal somehow gains sway and the protein is transcytosed. Interestingly, the receptor undergoes phosphorylation on a serine residue at approximately the time that it is delivered to the basolateral surface (Larkin *et al.*, 1986).

An anchor-minus construct of the polymeric immunoglobulin receptor was secreted from transfected MDCK cells into the apical medium (Mostov *et al.*, 1987). Another receptor construct, which lacks only the cytoplasmic tail, was also delivered directly to the apical membrane (Mostov *et al.*, 1986). Site-directed mutagenesis experiments which converted the phosphorylated serine residue into an alanine resulted in a protein which remained at the basolateral surface and was transcytosed extremely slowly (Casanova *et al.*, 1990). Converting the serine to an aspartic acid residue, however, resulted in a protein which was subject to rapid transcytosis.

Anyone encountering this hodgepodge of chimeras and truncations for the first time can be forgiven for feeling a pronounced urge to skip this section of the review. It is certainly not immediately obvious that any single thread might unify this rather disparate collection of experimental results. There are, however, theories which can explain many, if not all, of these observations and which are, therefore, worth considering. Perhaps the most aesthetically pleasing of these invokes the concept of a default pathway for membrane proteins. As was discussed above, the default, or unsorted, pathway need not necessarily lead to both cell surfaces. One can imagine, for example, a scenario in which only incorporation into an apically directed vesicle required a special sorting interaction for membrane proteins. According to this model, any protein not specifically pulled out of the stream of newly synthesized membrane proteins flowing through the TGN would be carried by bulk flow to the basolateral surface. Were this the case, the absence of a sorting signal would in itself serve as a sorting signal, directing basolateral localization. It should be noted that a cell which manifest a basolateral default pathway for membrane proteins could still possess an apical and basolateral default pathway for secreted proteins, since soluble polypeptides might be able to gain access to the lumenal space of apically directed carriers without need of a special signal.

If we go forward with the idea that the basolateral route corresponds to the default pathway (as has recently been proposed as well by Simons and

Wandinger-Ness, 1990), we can generate a scheme which is at least suffi-
ciently self-consistent to produce testable hypotheses. The postulates of
this proposal are as follows: (1) the VSV G protein contains no sorting
signal and is carried to the basolateral surface via the default pathway;
(2) the influenza HA protein contains an apical sorting signal in its ectodo-
main; and (3) a glycolipid anchor is sufficient to mediate apical targeting
and, therefore, glycolipid-anchored proteins carry no other sorting signals
(as has been suggested by Brown *et al.*, 1989). Were all of these axioms to
be true, one would expect that anchor-minus HA (which contains the
ectodomain's apical sorting signal) would be released apically, while
anchor-minus VSV G protein (which lacks a sorting signal) would be
released via the secretory default pathway to both surfaces. Furthermore,
an HA ectodomain–VSV G tail chimera would carry only an apical signal
and be delivered to the apical surface, whereas a G ectodomain–HA tail
chimera would bear no sorting signal and would follow the membrane
protein default pathway to the basolateral plasmalemma. Lipid-linked
VSV G ectodomain would travel to the apical surface by virtue of the
signal embedded in the lipid linkage. Anchor-minus PLAP, however,
which carries no signal, would be a candidate for the apically and baso-
laterally oriented secretory default release. Similarly, VSV G tail–PLAP
ectodomain hybrids would be signal-less and thus destined for basolateral
insertion.

Finally, this model can be expanded to integrate the polymeric immuno-
globulin receptor observations if it is assumed that the ectodmain of this
protein possesses an apical sorting signal which is unrecognizable so long
as the cytoplasmic tail exerts some inhibitory influence. Phosphorylation
of a serine residue on the tail would remove that inhibition and permit
expression of the apical targeting information. According to this formula-
tion, an intact and unphosphorylated receptor would display no recogniz-
able signal and would thus be shuttled by default to the basolateral surface.
Removal of the inhibitory tail, in either a tail-minus or anchor-minus
truncation, would result in the apical targeting of the remainder of the
polypeptide. Altering the phosphorylation site would prevent the tail's
inhibitory influence from being reversed and would condemn the mutant
protein to permanent residence in the basolateral plasmalemma. It should
be noted, however, that the results of experiments (described above) in
which the phosphorylated serine residue is converted to an aspartate
(which may preserve the negative charge) are difficult to reconcile with
this model. It may be that the signals and mechanisms involved in bio-
synthetic sorting differ from those associated with postendocytic sorting.
Were this the case, the complicated situation of the polymeric immuno-
globulin receptor could be explained more readily.

Evidence for this latter possibility comes from experiments in which the B1 and B2 isoforms of the macrophage Fc receptor were expressed in MDCK cells (Hunziker and Mellman, 1989). These two proteins are identical except for a 47-amino acid in-frame insertion in the cytoplasmic tail of the B1 polypeptide, which appears to diminish its capacity for endocytosis. The B1 form was found mostly apically, whereas B2 was predominantly detected in the basolateral domain. Both receptors could mediate IgG transcytosis in only the apical to basolateral direction. These results suggest that the steady-state distributions of these transcytotic receptors may reflect not only their handling by the biosynthetic sorting machinery but also their affinity for and interactions with elements of the endocytic apparatus.

The preceding scheme, although certainly prolix, is at least more or less consistent with many of the facts. Furthermore, the supposition of a basolateral default pathway is not without some theoretical justification. Most of the proteins commonly found in epithelial basolateral membranes are also present in the plasma membranes of nonpolarized cells. In contrast, apical membranes are generally endowed with "epithelia-specific" proteins. It could be argued that economy-minded Nature would design a system that would require sorting signals for only those proteins whose expression is limited to cells with more than one plasmalemmal destination. Confirmation or rejection of the model presented above will require elucidation of the membrane protein default pathway. Further clarification will require the identification and characterization of several membrane protein sorting signals as well as the identification of at least some of the cellular components which recognize and interpret these signals. Until these advances are achieved, models such as the one presented above, although unsatisfying, will have to suffice.

C. Sorting Mechanisms

If little is known of sorting signals, less is known about the mechanisms of sorting. None of the cellular components which participate in the targeting of newly synthesized epithelial membrane proteins have been identified. Very little has been learned or can be inferred about the routines which these putative components employ in carrying out their functions. Those who labor in the sorting field are inclined to think that this paucity of information derives from the inherent difficulty of the problem rather than from the inherent abilities of its investigators. In defense of this position, it should be noted that sorting has, until very recently, only been accessible to study in intact cells. *In vitro* (or semi-*in vitro* systems) capable of

carrying out bona fide sorting are only just being developed (Tooze and Huttner, 1990). It is not surprising, therefore, that the biochemical correlates of the sorting process have proved difficult to dissect.

The best understood sorting mechanism is the one which functions to target newly synthesized lysosomal enzymes from the Golgi complex to a prelysosomal compartment. A number of very elegant experiments have demonstrated that a family of receptors exists which recognize a mannose 6-phosphate residue added to the sugar structure of newly synthesized hydrolases during their passage through the Golgi (for review see Kornfeld, 1987). On binding these enzymes, the receptors transport them from the Golgi to a prelysosomal compartment whose acidic pH induces the dissolution of the receptor–hydrolase complex. Receptors unburdened of their ligand are free to return to the Golgi and to participate in another round of sorting. Weak bases such as NH_4Cl elevate the pH of the prelysosomal compartment and thus prevent release of the delivered enzyme. Since the complexed receptors cannot participate in further sorting, the cell's complement of available receptors is quickly exhausted and the newly synthesized hydrolases pass through the Golgi without being diverted to the lysosomal pathway. Thus, in the presence of this drug, newly synthesized lysosomal enzymes are secreted from the cell.

There is some reason to believe that an analogous model may be applicable to at least some aspects of other sorting phenomena. The requirement for sorting signals would certainly suggest the involvement of receptors which, by analogy with the lysosome system, might be expected to divert proteins into the appropriate pathways. Furthermore, the sorting of secretory proteins between the regulated and constitutive pathways in endocrine cells has been shown to require the participation of intracellular low-pH compartments (Kelly, 1985). Elevation of the pH of such compartments through the addition of weak bases such as NH_4Cl prevents sorting to the regulated pathway and results in the constitutive release of proteins which are normally stored intracellularly. It is tempting to think that low-pH compartments might subserve the same receptor regenerating function for regulated pathway sorting that they perform for the lysosmal enzymes.

Similar evidence has been gathered for proteins secreted constitutively from the basolateral surface of MDCK cells (Caplan et al., 1987). The basement membrane components laminin and heparan sulfate proteoglycan (HSPG) are normally released into the basolateral medium. In the presence of NH_4Cl, both of these proteins are released almost equally into both media compartments. Interestingly, the sorting of an apical secretory protein is unaffected by NH_4Cl. The basolateral targeting of the sodium pump is also not perturbed by this drug (Caplan et al., 1986) (see Fig. 4).
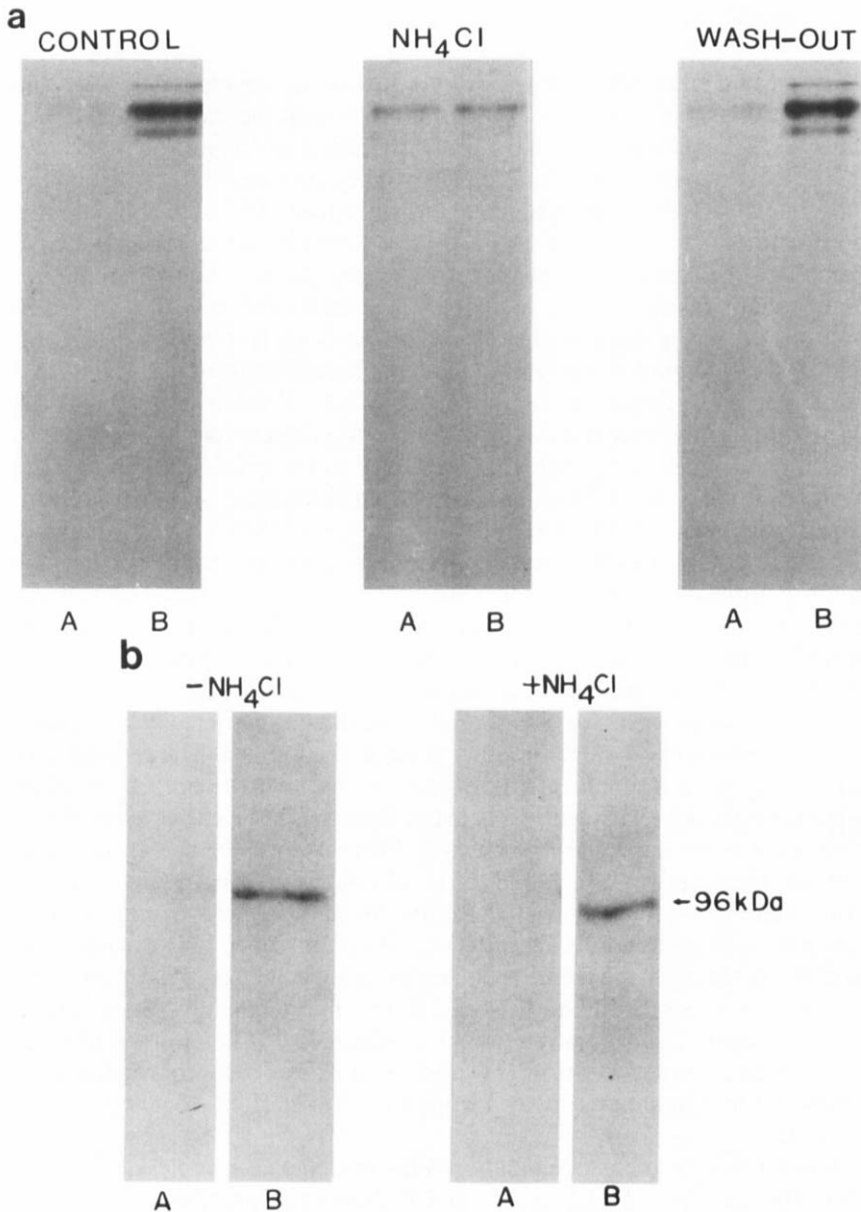
FIG. 4. A low-pH compartment is required for basolateral secretory, but not membrane, protein sorting in MDCK cells. MDCK cells grown on filters, as depicted in Fig. 2, secrete the basement membrane protein laminin into the basolateral medium (a, Control). In the presence of weak bases which elevate the pH of intracellular acidic compartments, laminin is released into both the apical and basolateral media (a, NH$_4$Cl). Removal of the weak base restores normal secretion (a, Wash-out). In contrast, the targeting of the newly synthesized Na$^+$/K$^+$-ATPase (assessed according to the protocol outlined in the text and Fig. 3) is unaffected by the presence of weak bases (b). Thus, distinct and pharmacologically separable mechanisms must operate in the sorting of these two proteins to the basolateral surface of MDCK cells. See text for details. (From Caplan *et al.*, 1986, 1987.)

If a lysosomal sorting-like model actually applies, it should be possible to identify the pH-sensitive receptor. A candidate for this function has been isolated from endocrine and exocrine cells (Chung *et al.*, 1989). This 25-kDa polypeptide appears to interact in a pH-sensitive fashion with only those proteins destined for packaging into regulated secretory granules. The degree to which this protein actually participates in sorting, however, remains to be established. Much less progress has been made in attempts to identify putative epithelial sorting receptors.

Finally, it should be noted that microtubules have been implicated as cellular components important in the epithelial sorting process. MDCK cells treated with microtubule-dissolving drugs deliver apical membrane and secretory proteins to both cell surface domains (Parczyk *et al.*, 1989; Rindler *et al.*, 1987). Interestingly, the basolateral delivery of membrane proteins is unaffected by the disruption of the microtubular network. Similar results have been gathered with intestinal epithelial cells (Achler *et al.*, 1989). It remains to be learned whether microtubules are functioning as tracks along which vesicles powered by microtubule motors are directed to their appropriate destinations or if they are subserving some other, as yet undefined function.

D. Generation and Maintenance of Epithelial Polarity

The discussion up to this point has focused on the sorting of membrane proteins in an already established epithelium. A closely related and extremely interesting problem is associated with the initial formation of a polarized epithelium *de novo* from nonpolarized cells. This process occurs during embryogenesis, where the nonepithelial early embryo gives rise to the highly polarized blastodermal epithelium. It also happens during the routine passage of polarized tissue culture cells, which involves the disruption of an established epithelial monolayer by trypsinization followed by the reorganization of a polarized cell layer subsequent to replating. A thorough discussion of the fascinating literature relating to these processes is beyond the scope of this review. Furthermore, it has been extremely well reviewed elsewhere (Rodriguez-Boulan and Nelson, 1989; Nelson, 1989). It is useful, however, to take note of some of the general themes which seem to be emerging from research in this field.

The generation of epithelial polarity requires cell–cell contact. MDCK cells plated on a substrate but prevented from forming cell–cell contacts are unable to generate fully polarized plasmalemmal domains (Gonzalez-Mariscal *et al.*, 1985; Nelson and Veshnock, 1987a; Vega-Salas *et al.*, 1987a). Conversely, MDCK cells grown in suspension but allowed to form

cell–cell contacts are able to organize at least partially polarized plasma-lemmal domains (Rodriguez-Boulan *et al.*, 1983; Wang *et al.*, 1990). In MDCK cells, the initiation of cell–cell contact has been shown to induce at least two effects. The first effect is the insertion into the plasma membrane of an intracellular structure called VAC, or vacuolar apical compartment. Vega-Salas *et al.* (1987b) have found that in MDCK cells denied cell–cell contact, apical membrane proteins accumulate in an intracellular compartment which is morphologically distinct from the organelles of the processing pathway. These fairly large vacuolar structures are frequently endowed with microvilli, which protrude into their lumena. The formation of cell–cell contacts induces the exocytosis of these structures, resulting in the insertion of pre–formed apical membrane at the cell surface. These observations suggest that, prior to the formation of an epithelium, the uniquely epithelial apical membrane cannot be expressed at the cell surface. Membrane traffic within the individual cells of a developing epithelium, therefore, appears to be directly responsive to influences from neighboring cells.

The second effect which has been recognized is the assembly of the cytoskeleton. The cytosolic surface of the basolateral plasma membrane of many epithelial cells (including MDCK) is covered by a cytoskeletal meshwork whose composition is remarkably similar to that of the erythrocyte cytoskeleton (Nelson, 1989). This web of ankyrin and fodrin (non-erythroid spectrin) appears to be bound to the membrane through interactions with trans-membrane proteins. The Na^+/K^+-ATPase has been shown to be one of the membrane anchors for this complex (Nelson and Veshnock, 1987b; Nelson and Hammerton, 1989; Morrow *et al.*, 1989), as has the cell adhesion molecule uvomorulin (Nelson *et al.*, 1990). In isolated MDCK cells denied cell–cell contact, the protein components of the cytoskeleton are disorganized and unassembled (Nelson and Veshnock, 1987a). Very quickly after the initiation of cell–cell contact, however, the basolateral cytoskeleton is formed. Nelson *et al.* (1990) have suggested that uvomorulin, which is involved in establishing cell–cell contact, communicates this event to the cytoplasm through its interaction with the cytoskeleton. According to this model, uvomorulin aggregates at sites of cell–cell contact, which in turn leads to the assembly of the cytoskeleton in these regions. The forming cytoskeleton essentially traps those proteins, such as the sodium pump, which are capable of interacting with it, leading to the generation of a biochemically differentiated membrane domain (McNeill *et al.*, 1990). Evidence from detergent extraction experiments (Salas *et al.*, 1988) suggests that interactions with an assembled, insoluble cytoskeleton may exist for both apical and basolateral proteins. While as yet unproven, this scheme offers an interesting and potentially testable

explanation for the epithelial cell's capacity to generate or regenerate a polarized state on interaction with its neighbors.

Once a polarized state is formed, the epithelial cell must be able to maintain it. Basolateral proteins must be prevented from diffusing into the apical domain and vice versa. The most important mechanism for preventing this intermixing is almost certainly the tight junction. Tight junctions have been shown to serve as barriers to the two-dimensional diffusion of both lipids and proteins (Cereijido *et al.*, 1989; van Meer, 1989; Dragsten *et al.*, 1981). Furthermore, disruption of tight junctions has been shown to allow randomization of epithelial plasmalemmal domains. The cytoskeleton may also play a role in preserving biochemical polarity. The involvement of the sodium pump with the cytoskeleton, for example, almost certainly curtails its mobility in the plane of the membrane (Jesaitis and Yguerabide, 1986).

Finally, endocytosis and postendocytic sorting must contribute to the stability of the polarized state. Fuller and Simons (1986) found that, following endocytosis, the basolateral transferrin receptor of MDCK cells was recycled to the basolateral membrane with greater than 99% fidelity. Matlin *et al.* (1983) found that basolateral proteins inserted into the apical surface (by fusing the VSV into the apical membrane) were efficiently removed and transcytosed to the basolateral plasmalemma. It seems clear, therefore, that the cell possesses mechanisms for continuously monitoring the compositions of its cell surface domains and for undertaking corrections should they be required.

IV. CONCLUSION

The cell surface membrane is the boundary between a cell and its environment. In the case of polarized epithelial cells, the apical plasma membrane is frequently the boundary between an organism and its environment. Consequently, the proteins that populate the plasmalemma play a tremendous role in determining the properties of individual cells and of whole tissues. Cells go to great trouble to regulate the compositions of their plasma membranes and to organize those membranes into subdomains capable of specialized functions. The processes through which this control and organization are created are the subjects of extremely active investigation. As promised in the introduction, this review has been long on description and speculation and short on the presentation of definitive mechanisms. I hope, however, that I have conveyed some of the excitement and complexity that attend this field and that I have succeeded in demonstrating its relevance to the membrane transport phenomena which are the subject of the remainder of this volume.

Acknowledgments

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