activated by agents that promote the breakdown of (poly)phosphoinositides, often with little change in intracellular free Ca\(^{2+}\). Furthermore, it has been shown that DG can induce structural transitions in phospholipid bilayers that are crucial to the necessary membrane function in vesicular exocytosis (Das & Rand, 1984). Hence, the B-50 protein may be involved in the control of exocytosis from those presynaptic terminals in which it is localized.

In this respect it should be noted that neuropeptides have been shown to act presynaptically as modulators of neurotransmitter release (Versteeg, 1980; Mulder et al., 1984; D. H. G. Versteeg, unpublished work). Previously, it was shown that ACTH and congeneres inhibit the phosphorylation of B-50 in rat brain synaptic plasma membranes (Zwiers et al., 1978; Gispen & Zwiers, 1985). The model presented concerning a role for B-50 as a feedback modulator in PtdIns4,5P\(_2\) hydrolysis, suggests that presynaptic modulation of neurotransmitter release by peptides may be brought about through changes in PtdIns4,5P\(_2\) metabolism (Gispen et al., 1985a,b).


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**Role of the Golgi complex and characteristics of post-Golgi transport in the biosynthesis of intestinal microvillar proteins**

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The small-intestinal enterocyte is a highly polarized cell which possesses a number of enzymes (mostly peptidases and glycosidases) functionally located in the apical (microvillar) portion of the plasma membrane (Kenny & Maroux, 1982). Due to the abundance of the microvillar enzymes, this cell type has become an attractive model system for studying eukaryotic plasma membrane protein biogenesis. Work performed in the last few years in several laboratories has revealed a common theme in the biosynthetic events of different microvillar enzymes. This includes a co-translational membrane insertion, high mannose glycosylation of the primary translation product in the rough endoplasmic reticulum and trimming and complex glycosylation of N-linked oligosaccharides (and probably O-linked glycosylation) in the Golgi complex before expression in the microvillar membrane (Danielsen et al., 1984). It has been unclear for some time, however, whether newly synthesized microvillar enzymes, as suggested by Quaroni et al. (1979) and Hauri et al. (1979), pass from the Golgi complex to the basolateral plasma membrane before insertion in the microvillar membrane. A related and more general problem, still unsolved, is at what stage the sorting of newly synthesized microvillar enzymes

Abbreviation used: SDS, sodium dodecyl sulphate.
from proteins with different subcellular destinations occurs. These two problems were tackled in the work presented here, the first by immunoelectron microscopy and by studying the orientation of aminopeptidase N (EC 3.4.11.2) in different membrane vesicles derived from labelled mucosal explants, the second by establishing an immunoelectrophoretic procedure for purification of membranes that harbour microvillar enzymes. In addition, the possible involvement of coated vesicles in the post-Golgi transport was studied.

Post-Golgi transport

Accessibility to proteinases. The orientation of newly synthesized aminopeptidase N in vesicles from the Mg⁺⁺-precipitated fraction (intracellular and basolateral membranes) and the microvillar fraction of labelled explants was studied by the enzyme's accessibility to proteolytic cleavage, as outlined in Fig. 1. Trypsin, proteinase K and papain cleaved most (72–90%) of the mature, complex-glycosylated form of \( M_r 160000 \), residing in microvillar vesicles, consistent with the fact that the bulk of the enzyme is exposed at the cell surface. In contrast, both the transient, high mannose glycosylated form of \( M_r 140000 \) as well as the mature form of aminopeptidase N from the Mg⁺⁺-precipitated fraction was largely protected from proteolytic cleavage (in the absence of Triton X-100), indicating an intravesicular location of both molecular forms. However, degradation products of both forms could be detected (Fig. 1). For the transient form, this minor susceptibility to proteolytic cleavage is most likely due to the presence of a small proportion of imperfectly sealed vesicles, as this form represents aminopeptidase N. In an early biosynthetic stage in the rough endoplasmic reticulum, i.e. in an exclusively intracellular and therefore ideally intravesicular, form. Since the relative amounts of undegraded transient and mature forms from the Mg⁺⁺-precipitated fraction were unchanged by treatment with any of the three proteinases, the two forms must have a similar orientation in the vesicles from this membrane fraction. However, involvement of the basolateral plasma membrane in the post-Golgi transport of aminopeptidase N to the microvillar membrane would imply that a proportion of the mature form of the enzyme resides in membranes of basolateral origin. In a study of the integrity and sidedness of basolateral vesicles, it has been shown that nearly 90% of these are in a right-side-out orientation (Boumendil-Podevin & Podevin, 1983) If aminopeptidase N resides transiently in this part of the plasma membrane, it would therefore be exposed at the vesicle surface and thus be accessible to proteolytic cleavage. However, the result of the proteolysis experiments argues against such vesicles being present in the Mg⁺⁺-precipitated fraction and consequently against the idea of the basolateral plasma membrane being the initial site for cellular externalization of aminopeptidase N.

Immunoelectron microscopy. The post-Golgi transport of aminopeptidase N has also been studied by immunoelectron microscopy. Here, immunogold labelling was concentrated in the microvillar membrane and was seen over the Golgi complex, whereas the basolateral plasma

Fig. 1. Outline of the experiments performed to determine the possible involvement of the basolateral plasma membrane in the post-Golgi transport of aminopeptidase N

The Mg⁺⁺-precipitated and microvillar fractions of explants, labelled for 80 min, were resuspended in 500 μl of 10 mM-Tris/HCl, pH 7.0, and incubated for 30 min at 20°C with 0.1 mg of either trypsin, proteinase K or papain (the latter pre-activated by incubation for 15 min at 37°C in the presence of 10 mM-cysteine)/ml. Control samples without addition of proteasine and, in the case of the Mg⁺⁺-precipitated fraction, a sample solubilized by Triton X-100 (2%), were incubated in parallel. After incubation, the samples were placed on ice and further proteolysis was inhibited by the addition of aprotinin (2.8 μg/ml), phenylmethylsulphonyl fluoride (0.1 mM) or iodoacetamide (5 mM). After solubilization with Triton X-100 (2%), the samples were quickly subjected to immunoelectrophoretic purification of aminopeptidase N. The gel tracks show the results obtained. The symbol (+) indicates proteinase K-treated; (−) indicates control; (T) indicates Triton X-100-solubilized before proteolysis.
Fig. 2. Localization of aminopeptidase N by immunogold staining and negative staining of purified coated vesicles

(a, d) Immunoelectron micrographs. Pig ileum mucosa was fixed in 2% glutaraldehyde and 3% paraformaldehyde, postfixed in 1% osmium tetroxide and embedded in epon. Thin sections (about 90 nm) were treated with 1% hydrogen peroxide for 5 min and carefully rinsed in TBS (50 mM-Tris/HCl, pH 7.4/150 mM-NaCl). To minimize non-specific antibody absorption the sections were floated in 10% normal sheep serum in gold buffer (50 mM-Tris/HCl, pH 7.0/150 mM-NaCl/1% bovine serum albumin/1% Triton X-100/0.02% sodium azide) before incubation with rabbit aminopeptidase N antibodies for 20 h at 4°C for 2 h at room temperature. After washing in gold buffer the sections were incubated with 5 nm or 20 nm gold-labelled goat anti-rabbit IgG for 1.5 h at room temperature, then washed in gold buffer (2 × 15 min), water (10 min) and finally contrasted in 1% aqueous uranyl acetate (30 min) and lead citrate (3 min). (a) An enterocyte brush-border. The labelling (5 nm granules) is evenly distributed all over the microvillar plasma membrane (MPM). Bar indicates 100 nm. (b) Apical portion of an enterocyte. The labelling (20 nm granules) is located in the microvillar plasma membrane (MPM), but no significant labelling can be seen in the basolateral plasma membrane (BPM). Bar indicates 500 nm. (c) Golgi complex (GO) showing labelling (5 nm granules) mainly on the trans-side of the organelle. Bar indicates 100 nm. (d) A vesicular structure (VE) in the apical portion of an enterocyte showing labelling (5 nm granules). Bar indicates 100 nm. (e) Electron micrograph of purified coated vesicles. Pig intestinal mucosa was homogenized and centrifuged at 20000 g for 30 min. The supernatant was centrifuged at 48000 g for 60 min to obtain a pellet of crude coated vesicles. This pellet was resuspended in Mes buffer (0.1 M-Mes, pH 6.0/1 mM-MgCl₂/0.5 mM-EGTA/0.02% sodium azide/0.1 mM-phenylmethanesulphonyl fluoride) containing 2% Triton X-100, washed, and centrifuged at 100000 g for 30 min. The supernatant was applied to a Sephacryl S-1000 column (1.6 cm × 90 cm), equilibrated in, and eluted with, Mes buffer containing 2% Triton X-100. Fractions containing purified coated vesicles were centrifuged at 48000 g for 90 min and the pellets finally resuspended in Mes buffer containing 0.1% Triton X-100. Pellets were negative-stained with 1% aqueous uranyl acetate. Bar indicates 100 nm. Insert: a clathrin cage; bar indicates 100 nm.
(a) Labelled mucosal explants

1. Homogenization in 1 ml of distilled water
2. Sonication for 2 x 1 min
3. Centrifugation at 12000 g, 10 min

Crude membrane preparation

1. Addition of 300 μl of 100 mM Na₂CO₃, pH 11.5
2. 30 min on ice, then addition of 100 μl of 1 M Tris/HCl, pH 7.5
3. Immunoelectrophoresis in 0.12% agarose
4. Excision and wash of precipitate
5. SDS/polyacrylamide-gel electrophoresis, fluorography

Fig. 3. Evidence of intracellular sorting of aminopeptidase N

(a) An outline of the procedure used for immunoelectrophoretic isolation of membranes harbouring microvillar enzymes. (b) SDS/polyacrylamide-gel electrophoretic analysis of membranes, isolated from explants that were labelled for 50 min. (1) A crude membrane preparation; (2-4) components precipitated with antibodies against aminopeptidase N (2), sucrase-isomaltase (3) and maltase-glucoamylase (4); (5) non-specifically aggregated material. After electrophoresis, the gel was prepared for fluorography. Apparent M, values (× 10³) are shown. (c) Densitometric scans of fluorographs of crude membrane preparations (bottom panels) and extracts from these (top panels) prepared by immunoprecipitation with antibodies against maltase-glucoamylase. The explants were labelled for 30 min (A), 50 min (B) and 80 min (C). '1' and '2' indicate the positions of the mature and transient forms of aminopeptidase N, respectively. Other peaks, likely to represent microvillar components, are marked by arrows.
membrane contained only insignificant amounts of labeling (Fig. 2a-d). In addition, smooth vesicles of 70-100 nm, positioned between the Golgi complex and the microvillar membrane, were labelled; these vesicles may well be responsible for the post-Golgi transport of newly synthesized microvillar enzymes. However, at present it cannot be ruled out that these vesicles might be endocytic and thus of apical origin.

Involvement of coated vesicles. In order to determine the involvement of coated vesicles in microvillar enzyme biogenesis, a method for preparing coated vesicles was established, yielding preparations judged to be ~99% pure by electron microscopy (Fig. 2e). Such preparations are analysed for contents of aminopeptidase N and sucrase-isomaltase (EC 3.2.1.20/EC 3.2.1.48). Immunoblotting experiments showed these enzymes to be present in their complex-glycosylated state but in a proteolytically cleaved form, indicating that they had been exposed to pancreatic proteases at the cell surface. This suggests that coated vesicles participate in the intracellular trafficking of endocytosed microvillar membrane whereas it is unclear whether they also transport newly synthesized enzymes to the microvillar membrane.

In summary, we propose that cells with a polarized plasma membrane possess two different post-Golgi pathways for expression of protein components in the two domains of the plasmalemma. Admittedly, the data presented do not entirely rule out an extremely rapid transfer through the basolateral plasma membrane, nor the possibility of a minor proportion of newly synthesized microvillar enzymes being lost to this membrane. Both cases would render the steady-state pool of labelled aminopeptidase N undetectable in this compartment. However, a half-life in the basolateral membrane fraction of about 30 min, as was observed for rat sucrase-isomaltase (Hauri et al., 1979), would clearly be detectable. Recent work on viral protein biogenesis in infected Makin-Darby canine kidney cells indicates that haemagglutinin of apically budding influenza virus, like microvillar enzymes, follows a direct pathway leading from the Golgi complex to the apical surface (Matlin & Simons, 1984; Misek et al., 1984; Rodriguez-Boulan et al., 1984; Rindler et al., 1985).

Sorting of newly synthesized microvillar enzymes

Immunoelectrophoretic isolation of membranes harbouring microvillar enzymes. This technique was established as a method for studying the sorting involved in the accumulation of microvillar enzymes in areas of the membrane from which other proteins are excluded. The rationale for this approach is that, before any sorting, the newly synthesized enzymes are too diffusely distributed in the membrane to form a basis for an immunopurification of the membranes harbouring them. As a result of sorting, however, their density in some areas of the membrane might increase (Griffiths et al., 1984; Quinn et al., 1984) and thereby generate a sufficiently high number of antigenic recognition sites for immunoprecipitation. The rather long time preceding its microvillar expression (about 60 min) made aminopeptidase N a suitable probe for this study. In addition, since this enzyme represents about 1.5% of the total protein synthesis in the enteroocyte (Danielsen, 1984), aminopeptidase N is a major component whose transient and mature forms predominate and are easily recognized bands in the high-M<sub>r</sub> region of SDS/polyacrylamide gels.

Alkaline treatment (Fujiki et al., 1982) of a crude membrane preparation before immunoelectrophoresis was performed to open up intracellular vesicles and thus expose microvillar-destined enzymes (Fig. 3). Membranes were precipitated with antibodies against aminopeptidase N, sucrase-isomaltase or maltase-glucoamylase (EC 3.2.1.20). In the absence of antibodies, only small amounts of membrane material were arrested in the gel, indicating that the precipitation was caused by the antibodies.

Membranes from explants, labelled for periods of 30-80 min, were immunoprecipitated according to this procedure. After 30 min of labelling, the transient form of aminopeptidase N appeared as a distinct band but was only moderately enriched (about two-fold), compared with the crude membrane preparation (Fig. 3). Much more significant, however, was the enrichment of the mature form of the enzyme; this was estimated to be at least 30-fold. In addition, many other polypeptides were precipitated. One, of M<sub>r</sub> 240 000, represents the transient and mature forms of both sucrase-isomaltase and maltase-glucoamylase, and some, of M<sub>r</sub> 40 000 (actin), M<sub>r</sub> 95 000 (villin) and M<sub>r</sub> 105 000, probably correspond to microvillar cytoskeletal components (Cowell & Danielsen, 1984). Other bands probably correspond to co-precipitated proteins of various intracellular membranes.

The profiles of the crude membrane preparations and the immunoprecipitated fractions after 50 min of labelling resembled those obtained after 30 min. A larger proportion of aminopeptidase N had now undergone conversion to the mature form in the crude membrane preparation, but the enrichment of this molecular form by the immunoprecipitation was still considerable. After 80 min of labelling, the proportions of transient and mature forms of aminopeptidase N in the crude membrane preparation were approximately the same, but in the immunoprecipitated fractions the proportion of the transient form was drastically decreased. This agrees well with the finding that microvillar membranes are the major constituent of the immunoprecipitated material (data not shown) and therefore, when labelled, become the predominant pattern in the fluorograph.

The poor enrichment of the transient form of aminopeptidase N indicates that at the pre-Golgi stage, most of the enzyme molecules are too scattered in the membranes of the endoplasmic reticulum to become effectively precipitated by the immunoelectrophoretic procedure. In contrast, there was considerable enrichment of the small proportion of aminopeptidase N that after 30 and 50 min of labelling had undergone processing to yield the mature form. The significant difference in enrichment between the two molecular forms of the enzyme makes it reasonable to assume that sorting occurs concomitantly with, or shortly after, the conversion from transient to mature form, i.e. in the Golgi complex or soon after exit from this organelle. This interpretation is supported by the immunoelectron microscopic studies, where aminopeptidase N first achieved a density in the membrane, sufficient for detection, in the Golgi complex. The underlying mechanism whereby this sorting occurs remains unknown, but is an attractive subject for further study.
Hepatic endosomes: preparation, properties and roles in receptor recycling

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It is now clear that many plasma membrane proteins, especially receptors, constantly move into and out of the interior of mammalian cells. Aspects of this trafficking of membrane proteins have been explored by monitoring the uptake (endocytosis) of a variety of ligands that attach specifically to cell-surface receptors. In the hepatocyte, the wide range of endocytosed ligands has provided opportunity for tracing the internalization pathways, and these include circulating proteins (transferrin, caeruloplasmin), antibodies (polymeric IgA), polypeptide hormones, growth factors, transmitters, toxins and viruses. The trafficking of many surface receptors appears to be a continual and constitutive property of cells, shown to proceed in the absence of bound ligands, e.g. polymeric IgA (Mullock et al., 1984) and transferrin (Watts, 1985).

Endocytosis is a regulated process for delivering the various ligands to specific intracellular loci. On the one hand, ligands such as toxins, β-adrenergic transmitters and many asialoglycoproteins are transferred within minutes to the lysosomal compartment for degradation, whereas others, such as some polypeptide hormones and growth factors and viruses, are able to evade or delay immediate transfer to the lysosomes. Other ligands are returned to the cell surface after brief residence in the endocytic network, and in hepatocytes, released either into blood (transferrin, asialotransferrin) or bile (polymeric IgA).

The pathways of endocytosis have been explored mainly by using biochemical or morphological techniques. Biochemical techniques are being applied not only in tracing the subcellular pathways followed by ligands as they migrate into the cell from the plasma membrane, but crucially in identifying, isolating and characterizing the membrane compartments traversed. At first, it was thought that the transfer of ligands from the plasma membrane to intracellular destinations could be accommodated within the framework of either the lysosomal or Golgi apparatus membrane systems. Investigations, using a variety of cell types, located ‘tagged’ ligands in membrane vacuoles in the vicinity of lysosomes or in the precursors of the Golgi apparatus (Farquhar, 1983; Steinman et al., 1983). With the demonstration of roles for the intermediary compartments in dissociation of ligand–receptor complexes, and their selective sorting, these membranes now constitute a functionally and biochemically distinctive organelle. In a field where progress has resulted from subcellular fractionation, autoradiography and immunochemistry, it is inevitable that a number of terms and acronyms have been used to describe the components of the endocytic compartment, including receptosomes, CURL (compartment for uncoupling receptors and ligands), ligandosomes, diacytosome, intermediary vesicles, pinosomes, etc. However, the term ‘endosomes’ is now widely used to describe the membrane vacuoles and tubular extrusions seen in vivo and in subcellular fractions where the ligands are located after transfer from the plasma membrane, and so will be used in this review.

Preparation and subfractionation of endosomes
First clues as to the physical properties of endosomes were obtained when subcellular components, containing various intact radioiodinated ligands internalized by perfused rat livers, were shown to equilibrate at low-density positions when centrifuged in sucrose gradients (Smith et al., 1980). Subsequently a preparative technique was developed that used asialotransferrin type 3, insulin and prolactin as radioiodinated markers of high specific activity to identify and purify the endosomes at early (1–2 min), and later (10–15 min) periods after uptake by rat liver (Debanne et al., 1982; Evans et al., 1983; Evans, 1985; Saermark et al., 1985). These endosome fractions (see Fig. 1 for method of preparation) were shown, by morphological and enzymic criteria, to differ in properties from plasma membranes, lysosomes and endoplasmic reticulum. However, the presence of galactosyl- and sialyl-transferase activities in the fractions containing high specific activities of radioiodinated ligands was