REVIEW ARTICLE

Biosynthesis of microvillar proteins

E. Michael DANIELSEN, Gillian M. COWELL, Ove NORÉN and Hans SJÖSTRÖM

Department of Biochemistry C, The Panum Institute, University of Copenhagen, Copenhagen, Denmark

Introduction

Generally, the motivation for studying the biosynthesis of plasma membrane proteins lies in the importance of the functions these proteins may exhibit, be it cell-to-cell communication, the ability to assimilate fuels and other compounds for the cell, or the transmission/reception of signals. At the forefront of this field is work on membrane-enveloped viruses such as the vesicular stomatitis virus and Semliki Forest virus (Garoff *et al.*, 1982). However, in order to assess the general validity of the models emerging from research on the viral systems, it is important to study the biosynthesis of the endogenous eukaryotic plasma membrane proteins. During the last few years, such information has been obtained for the histocompatibility antigens (Dobberstein *et al.*, 1979; Krangel *et al.*, 1979; Owen *et al.*, 1980) and the erythrocyte membrane proteins (Jokinen *et al.*, 1981; Braell & Lodish, 1981). In addition to a few reports on the liver plasma membrane proteins (Bergeron *et al.*, 1975; Elovson, 1980), the information on biosynthesis of endogenous plasma membrane proteins of polarized epithelial cells has come from studies on intestinal and kidney microvillar proteins. The small intestinal enterocyte and the kidney proximal tubule cell both possess a distinct brush border of closely packed microvilli. This luminal part of their plasma membrane is demarcated from the basolateral membrane by tight junctions and contains several integral membrane proteins in substantial amounts. Many of these have been purified (Table 1) and their structure and function have been under intensive study during the last decade (for a review, see Kenny & Maroux, 1982). In addition, specific antibodies, polyclonal (for references see Danielsen, 1983) as well as monoclonal (Hauri *et al.*, 1980; Gee *et al.*, 1983) have been raised against these enzymes. Furthermore, effective methods for isolating microvillar membranes from both intestine and kidney have been described (Schmitz *et al.*, 1973; Booth & Kenny, 1974), as have methods for organ culture of tissue explants for long enough periods to study biosynthetic events (Moscona *et al.*, 1965). Lately, preparations of translatable mRNA from both small intestine (Wacker *et al.*, 1981; Danielsen *et al.*, 1982b) and

kidney (Nash & Tate, 1984) have become available, and thus the scientific tools and model systems necessary for a thorough study of the biosynthesis of microvillar proteins are at hand.

As mentioned above, an essential property of both the intestinal enterocyte and the kidney proximal tubular cells is the ability to achieve and maintain a polarized plasma membrane. In this context, an insight into the mechanisms and events responsible for the expression of the microvillar enzymes in the apical portion of the plasma membrane is central to the understanding of the function of these cells. This implies an elucidation of (1) the intracellular route of transport used by the cell to transfer newly synthesized enzymes to their site of function; (2) the nature of the processing of the primary translation products, yielding the functional enzymes; and (3) the sorting mechanism(s) discriminating between proteins destined for insertion in the microvillar membrane or other cellular compartments such as the endoplasmic reticulum, the Golgi complex, the lysosomes and the basolateral plasma membrane. The fact that the microvillar membrane harbours so many different enzymes provides a special opportunity to compare the biosynthetic events of these, and thereby to obtain a more generalized picture of the biogenetic process. If supplemented with similar data for the cytoskeletal proteins responsible for the structure of the microvilli, this picture could provide a basis for the understanding of the assembly of the highly ordered structure of a microvillus. Although the problems in achieving this goal are plentiful, the interest devoted to this field by various research groups has yielded considerable information from which a general pattern now evolves. The aim of this Review is to compile and interpret the results obtained so far and to discuss them in the light of advances made in related fields of cell biology.

Translational events

Cellular site of polypeptide synthesis

A central question of relevance for problems concerned with biosynthesis is at what stage during this process the microvillar enzymes become associated with a membrane. In the study of other types of membrane and secretory proteins, several

different cellular membranes have been shown to be 'translocation competent' (Blobel, 1980), i.e. capable of mediating the transfer of newly synthesized polypeptides across the lipid bilayer. However, the various translocation-competent membranes differ principally in the way they exert their functions. Thus, a co-translational translocation mode is characteristic of, for instance, the rough endoplasmic reticulum and the prokaryotic plasma membrane, whereas mitochondrial and chloroplast membranes function in a post-translational mode (Blobel, 1980). An alternative theory, which does not emphasize the temporal relationship between polypeptide synthesis and membrane translocation, the 'membrane trigger' hypothesis, has been proposed by Wickner (1979).

For the microvillar enzymes, the data obtained clearly indicate that the membrane insertion occurs well before the newly synthesized enzymes appear in the microvillar membrane. A membrane-bound intracellular form of *in vivo* labelled rat sucrase-isomaltase was first demonstrated by Hauri *et al.* (1979). In an organ culture system of pig intestine (Danielsen *et al.*, 1982a), aminopeptidases N and A, dipeptidyl peptidase IV, sucrase-isomaltase, maltase-glucoamylase and lactase-phlorizin hydrolase, labelled with a 10 min pulse of [³⁵S]methionine, were all exclusively located in a Ca²⁺-precipitated membrane fraction rather than occurring in soluble form (Danielsen, 1982; Danielsen *et al.*, 1983a, E. M. Danielsen, unpublished work; Sjöström *et al.*, 1983). A similar result has been obtained for the pig kidney aminopeptidases N and A, dipeptidyl peptidase IV and endopeptidase-24.11 (J. R. Stewart & A. J. Kenny, personal communication) and for rat kidney γ -glutamyl transpeptidase (Nash & Tate, 1982). Thus, a cellular membrane different from the microvillar membrane must be translocationally active in the membrane insertion of these enzymes. In the case of eukaryotic cells, the principal translocation-competent membrane (for non-mitochondrial proteins) is the rough endoplasmic reticulum. This organelle, as mentioned above, is believed to function in a co-translational manner, but the surprising results of Cezard *et al.* (1979) and Maze & Gray (1980), reporting the existence of soluble precursor forms of rat sucrase-isomaltase and aminopeptidase N, argued in favour of a post-translational membrane insertion.

The interaction of the rough endoplasmic reticulum with nascent polypeptides destined to become microvillar enzymes is difficult to study under conditions resembling those *in vivo*, owing to a rapid further processing of the primary translation products. A more suitable experimental system for this is cell-free translation of intestinal or kidney mRNA, where the influence of the rough

endoplasmic reticulum [in the form of rough microsomes, usually obtained from dog pancreas (Jackson & Blobel, 1977)] can be observed under well-defined conditions. So far, experiments of this type have been performed for rabbit sucrase-isomaltase (Wacker *et al.*, 1981), pig intestinal aminopeptidase N (Danielsen *et al.*, 1983b) and rat γ -glutamyl transpeptidase (Nash & Tate, 1984). In these cases, the newly synthesized microvillar enzyme was found predominantly in the membrane fraction of the translation mixture, indicating an interaction between the microsomal membranes and the polypeptide synthesized. For aminopeptidase N and γ -glutamyl transpeptidase, a concomitant, membrane-dependent N-linked high-mannose glycosylation of the primary translation product was demonstrated and is indicative of a membrane translocation, since this type of processing is known to occur exclusively on the luminal side of the endoplasmic membrane (Snider & Robbins, 1982). For γ -glutamyl transpeptidase, this orientation was further confirmed by protection from proteolytic digestion in the presence of microsomal membranes. In the case of both aminopeptidase N and γ -glutamyl transpeptidase, the insertion into microsomal membranes took place only when these were present during translation; post-translationally added microsomes did not interact with, nor process, the primary translation products. It can therefore be concluded that the data obtained from cell-free translation experiments argue in favour of a co-translational membrane insertion of the microvillar enzymes, taking place in the rough endoplasmic reticulum.

Size of the primary translation product

The finding that the A-subunit of pig intestinal, but not renal, aminopeptidase N is cleaved *in situ* into B- and C-subunits by proteinases under physiological conditions (Sjöström *et al.*, 1978) was indicative that the mature subunit structure of microvillar enzymes does not necessarily reflect the size of their primary translation products. The existence of large, single-chain precursor polypeptides was first demonstrated for rat (Hauri *et al.*, 1979) and pig (Sjöström *et al.*, 1980) sucrase-isomaltase. Analogous precursors have since been shown to exist for pig maltase-glucoamylase (Sørensen *et al.*, 1982), pig and human lactase-phlorizin hydrolase (E. M. Danielsen, unpublished work; Sjöström *et al.*, 1983; Skovbjerg *et al.*, 1984) and rat kidney γ -glutamyl transpeptidase (Nash & Tate, 1982). In the case of sucrase-isomaltase, it is evident that its two functionally different subunits are translated from a two-cistron mRNA, possibly generated during evolution by a gene duplication (Semenza, 1979). It is tempting to speculate that maltase-glucoamylase resembles sucrase-iso-

maltase in this respect and possibly also lactase-phlorizin hydrolase, though in the latter case it is unknown if the two equally sized subunits are structurally and functionally different. In contrast, the subunit M_r of intracellular precursor forms of pig intestinal aminopeptidase A and dipeptidyl peptidase IV (Danielsen *et al.*, 1983a) and pig kidney aminopeptidases N and A, dipeptidyl peptidase IV and endopeptidase-24.11 (J. R. Stewart & A. J. Kenny, personal communication) are comparable to the size of the corresponding mature subunits. Though these enzymes may conceivably be derived from larger precursors, difficult to detect because of an early (co-translational) cleavage, the evidence so far argues that the identical subunits of these enzymes are generated independently.

Does signal cleavage occur during the biosynthesis of microvillar enzymes?

It is now widely accepted that the structural requirement ensuring membrane translocation of secretory as well as membrane proteins resides in a stretch of 15–20 largely hydrophobic amino acids on the primary translation product (Sabatini *et al.*, 1982). This 'signal' mediates the contact between the polypeptide-synthesizing ribosomal complex and the relevant translocating membrane, for some proteins shown to be assisted by a specific 'signal recognition particle' and a 'docking' protein (Meyer, 1982). For the majority of both secretory and membrane proteins studied, the signal is located in the *N*-terminal region of the primary translation product and is cleaved during or shortly after polypeptide synthesis. It is now recognized, however, that neither cleavage of, nor the *N*-terminal position of, the signal is essential for the membrane translocation (Blobel, 1980; Sabatini *et al.*, 1982).

Pig intestinal aminopeptidase N is the only microvillar enzyme where the position of the signal has been studied (Danielsen *et al.*, 1983b). Synchronous translation experiments were used to determine the approximate position of the signal along the primary translation product. When translation was performed at 22°C, where 75 min are required to complete synthesis, microsomal membranes needed to be added no later than 10–20 min after initiation to ensure proper membrane translocation, thus placing the signal in the *N*-terminal quarter of the polypeptide. If the folding of the nascent polypeptide of this relatively large protein proceeds slowly, it is likely that the signal is located very close to the *N*-terminus. The similarity in size (within the resolution of the gel system, 1500 Da) of the primary translation product, determined in the absence of microsomal membranes, and a molecular form of the enzyme obtained from

tunicamycin-exposed intestinal explants may suggest that the signal does not undergo cleavage during membrane translocation. This finding, albeit provisional until confirmed by stricter experiments such as radiosequencing, bears some topological consequences for aminopeptidase N. Like other microvillar enzymes studied, aminopeptidase N is anchored to the membrane by a small hydrophobic segment near its *N*-terminus which spans the lipid bilayer (Kenny & Maroux, 1982), possibly giving the enzyme a 'bitopic' membrane configuration in the terminology of Blobel (1980). Aminopeptidase N may therefore be the first example of a bitopic membrane protein with a non-cleaved signal (Fig. 1a). This would mean that the signal sequence has a permanent function in this enzyme, namely to provide an anchoring of the protein to the membrane, as envisaged by Kenny & Booth (1978). A similar conclusion was recently reached for rat kidney γ -glutamyl transpeptidase, in which case the cell-free translation product (in the absence of microsomal membranes) was found to be slightly smaller than an endoglycosidase H-treated, high-mannose glycosylated form of the enzyme (the small size difference could be accounted for by residual carbohydrate on the latter form) (Nash & Tate, 1984). Whereas the linear model of membrane insertion (Blobel & Dobberstein, 1975) does not easily explain this configuration, it fits well with the alternative loop model (Inouye & Halegoua, 1979; Engelman & Steitz, 1981) as pointed out by Desnuelle (1979). This model, if correct, might be applicable to other microvillar enzymes, but it is interesting to note that sucrase-isomaltase, which also contains basic amino acids in its *N*-terminal region (Sjöström *et al.*, 1982; Hauri *et al.*, 1982), seems to have an ectoplasmic *N*-terminus (Frank *et al.*, 1978). Assuming the loop model of protein translocation, this would suggest the existence of a cleavable signal on the primary translation product of sucrase-isomaltase (Fig. 1b).

Intracellular route of transport

Microscopic studies

Early studies devoted to the problem of intracellular transport investigated the movement of glycoproteins, labelled *in vivo* with various radioactive carbohydrates, through different cellular organelles (for a review see Kenny & Maroux, 1982). Radioautographs from such experiments revealed an initial labelling of the Golgi complex followed by a labelling of other cellular compartments, including the microvillar and basolateral portions of the plasma membrane and lysosomes. A similar flow of newly synthesized glycoproteins was also observed in pulse-chase labelling studies, using subcellular fractionation (Quaroni *et al.*,

Fig. 1. Possible mechanisms for membrane insertion of microvillar enzymes

(a) The polypeptide transverse the membrane in one loop, yielding a bitopic configuration with an endoplasmic *N*-terminus, carrying a permanent signal. (b) The polypeptide transverse the membrane in two loops, yielding a polytopic configuration. An ectoplasmic *N*-terminus implies that the signal has been cleaved (arrow).

1979a). However, with the advent of antibodies with specificity towards individual microvillar enzymes it has become technically possible to study the biogenesis of these separately. In the elucidation of the transport route used by eukaryotic cells to express viral proteins, the radioautographic detection of newly synthesized antigen by a specific antibody, coupled to an electron-dense marker, has proved a powerful technique (Green *et al.*, 1981; Bergmann *et al.*, 1981). Only recently have attempts to apply this approach to the microvillar enzymes been reported (Lorenzson *et al.*, 1983). This has undoubtedly to do with the fact that these enzymes, contrary to viral proteins, each represent only a tiny portion (0.1–1%) of the total mass of protein of the cell and that their biosynthesis therefore inevitably must be studied against a high background. By using immunofluorescence labelling, Feracci *et al.* (1982) detected rabbit and pig intestinal aminopeptidase N in a structure tentatively identified as the Golgi complex. In the same work, this enzyme could also be localized under the terminal web and in the upper part of the lateral plasma membrane.

Cell-fractionation studies

Most of the results obtained on intracellular transport of microvillar enzymes are based on biochemically designed experiments, including subcellular fractionation and protein purification. A fractionation procedure that has proved to be of particular use is the Ca^{2+} - or Mg^{2+} -precipitation technique, where divalent cations are used to aggregate intracellular membranes as well as the basolateral membrane, making an isolation of microvillar membranes (in vesiculated, right-side-out, form) easy and rapid. The technique works equally well with both intestinal (Schmitz *et al.*, 1973) and kidney (Booth & Kenny, 1974) tissue and has been used in preparative scale in the purification of several microvillar enzymes (Kenny &

Maroux, 1982). Using a modification of the procedure of Kessler *et al.* (1978), it was possible to obtain a Ca^{2+} -precipitated membrane fraction that contains only 2–4% of the total activity of the microvillar enzymes, indicating a minimal contamination with microvillar membranes (Danielsen *et al.*, 1981a). Several enzymes, purified by immunoabsorbent chromatography from Ca^{2+} -precipitated membrane fractions of pig intestine, were found to have a molecular form different from those of the corresponding enzymes obtained from microvillar membranes (Danielsen *et al.*, 1981a,b). The new molecular forms were structurally related to the mature enzymes, as they formed precipitates in immunoelectrophoresis against antibodies directed towards the latter. Some of these molecular forms that were of lower M_r were found to be high-mannose glycosylated, as judged by their sensitivity to endoglycosidase H, whereas others were resistant to this glycosidase but sensitive to neuraminidase. These studies supported the idea of a membrane-bound intracellular transport of microvillar enzymes with concomitant processing of the carbohydrate moiety. The kinetics of this transport have been studied by pulse-chase labelling of pig intestinal explants in organ culture (Danielsen, 1982; Danielsen *et al.*, 1983b; E. M. Danielsen, unpublished work; Sjöström *et al.*, 1983). Aminopeptidases N and A, dipeptidyl peptidase IV, sucrase-isomaltase, maltase-glucoamylase and lactase-phlorizin hydrolase all appeared in the Ca^{2+} -precipitated membrane fraction after a 10 min pulse of [^{35}S]methionine as high-mannose glycosylated polypeptides. This form prevailed for 40–60 min and thereafter a conversion to the complex glycosylated form took place, in the Ca^{2+} -precipitated membrane fraction. The appearance of these enzymes in the microvillar membrane occurred shortly after this conversion, after about 60–90 min of chase. Results parallel to these have been obtained for pig kidney aminopeptidases N and A,

dipeptidyl peptidase IV and endopeptidase-24.11 by pulse-chase labelling of kidney slices (J. R. Stewart & A. J. Kenny, personal communication). Comparable kinetics of transport through cellular membranes followed by expression in the microvillar membrane have also been reported for rabbit intestinal sucrase-isomaltase (Hauri *et al.*, 1979), rat kidney γ -glutamyl transpeptidase (Nash & Tate, 1982) and for rat intestinal aminopeptidase N (Ahnen *et al.*, 1982, 1983). It is therefore reasonable to propose that a common, membrane-bound route of transport exists for the transfer of microvillar enzymes from their site of synthesis, the rough endoplasmic reticulum, to their final destination. Furthermore, since the lag period preceding the appearance in the microvillar membrane is compatible with that preceding the externalization of secretory proteins, it is tempting to argue that the biogenesis of microvillar enzymes follows the general 'membrane flow' theory of Palade (1975).

A more refined subcellular fractionation than precipitation with divalent cations is required to resolve the various membrane compartments and to study their possible involvement in the transport of newly synthesized microvillar enzymes. Using equilibrium gradient centrifugation, Hauri *et al.* (1979) were able to demonstrate a flow of [3 H]fucose-labelled rat sucrase-isomaltase from the Golgi complex, via the basolateral plasma membrane, to the microvillar membrane. A similar route of transport was recently suggested for rat kidney γ -glutamyl transpeptidase by Kuno *et al.* (1983). In contrast, Ahnen *et al.* (1982, 1983), studying the intracellular transport of [3 H]leucine-labelled rat intestinal aminopeptidase N, observed a flow of the enzyme from endoplasmic reticulum/Golgi membranes directly to the microvillar membrane. These authors concluded that the apparent incorporation into the basolateral plasma membrane could be accounted for by contamination from the endoplasmic reticulum. These apparently conflicting results may reflect the difficulties that exist in obtaining pure and well-defined membrane fractions by classical separation techniques. Mircheff *et al.* (1979) have emphasized the difficulties of separating smooth-surfaced membranes from the enterocyte, and have used special measures, such as free-flow electrophoresis or density perturbation by digitonin, to separate basolateral plasma membranes effectively from smooth endoplasmic membranes.

Studies with specific inhibitors

A different approach is to study the effect on transport of drugs with known inhibitory functions. When pig intestinal explants were labelled in the presence of monensin, which impairs the func-

tions of the Golgi complex (Tartakoff, 1982) and the endocytic vacuoles and lysosomes (Marsh *et al.*, 1982) without interfering with protein synthesis, the intracellular traffic of newly synthesized enzymes to the microvillar membrane was arrested (Danielsen *et al.*, 1983c). Concomitant with this, an inhibition of polypeptide processing was observed (discussed below), together with a progressively developing vacuolization of the Golgi complex. The antimicrotubular agent colchicine, known to interfere with the transport of newly synthesized glycoproteins in the intestine of rat (Quaroni *et al.*, 1979b) and human (Blok *et al.*, 1981a), was found also to inhibit the transport of pig intestinal enzymes to the microvillar membrane (Danielsen *et al.*, 1983c). Since biosynthesis and processing of the enzymes were unaffected by colchicine, it was suggested that microtubules may be active in the final transport from the Golgi complex to the microvillar membrane.

Different kinetics of transport

Gray *et al.* (1983), comparing the kinetics of intracellular transport of newly synthesized rat intestinal aminopeptidase N and sucrase-isomaltase, observed that the former appears earlier in the endoplasmic reticulum and is also more rapidly transferred to the Golgi complex. It was hypothesized that the differential rate of transport might be due to separate vesicular pathways for the two enzymes. We have likewise observed that the larger polypeptides of pig sucrase-isomaltase and maltase-glucoamylase are more difficult to detect than aminopeptidase N immediately after a 10 min pulse (Danielsen, 1982; Danielsen *et al.*, 1983a). However, comparison of densitometric scans of fluorographs of these three enzymes after 3 h of continuous labelling showed an approximately similar distribution between the Mg^{2+} -precipitated membrane fraction and the microvillar fraction, arguing against the existence of separate, kinetically different transport routes for the individual microvillar enzymes (E. M. Danielsen, unpublished work). Still, minor variations among the enzymes are indeed possible and could conceivably be caused by a differential affinity for the transporting and sorting systems, operating between the organelles responsible for processing, and the microvillar membrane.

In contrast to the considerable lag period preceding the expression of enzymes in the microvillar membrane, other protein components appear very rapidly in the microvilli. Thus, when pig intestinal explants were pulsed for 10 min with [35 S]methionine, several labelled polypeptides were immediately detectable in the microvillar fraction (Cowell & Danielsen, 1984). None of these bands could be labelled by [3 H]fucose, and from the experiments

using an alkaline extraction technique (Fujiki *et al.*, 1982) it was concluded that the rapidly labelled bands were of cytoskeletal origin and not integral membrane proteins. One of these, of M_r 42000, was presumed to be actin and two other bands of M_r 110000 and 95000 reacted with antibodies to the 110 kDa protein and villin, respectively. These findings indicate that the membrane proteins and cytoskeletal components of the microvilli are synthesized and transported to their common destination at different rates and by separate mechanisms.

Possible post-Golgi pathways and mechanisms for sorting

The only cellular membrane compartments whose involvement in the transport of newly synthesized microvillar enzymes have been reasonably ascertained are the endoplasmic reticulum and the Golgi complex. The uncertainty about the basolateral plasma membrane arises from the lack of knowledge about the pathway(s) operating between the Golgi complex and the microvillar membrane. However, the detection of small amounts of immunoreactive aminopeptidase N in the basolateral plasma membrane (Colas & Maroux, 1980) is at least consistent with the idea that this membrane may be active in the transport of microvillar enzymes (Quaroni *et al.*, 1979a). Microvillar glycoproteins have also been detected in the lysosomes, where their presence has been ascribed to crinophagy, i.e. a degradative, regulating pathway for newly synthesized enzymes (Blok *et al.*, 1981a,b).

Much more information on traffic in the apical part of polarized cells has been obtained from studies on endocytosis, and it may be relevant briefly to state the known routes of traffic. (1) Transport from the microvillar membrane (via the endosomes?) to the basolateral plasma membrane (Rodewald & Abrahamson, 1982; Matlin *et al.*, 1983). (2) Recycling of microvillar proteins (via the endosomes?) (Louvard, 1980). (3) Transport (via endosomes?) to the lysosomes (Rodewald & Abrahamson, 1982). In addition, active sorting occurs, probably in the acid endosome compartment (Rodewald & Abrahamson, 1982; Matlin *et al.*, 1983). It would be interesting to know if the pathways for endo- and exocytosis coincide. Transport in MDCK cells of newly synthesized influenza virus hemagglutinin is inhibited in a post-Golgi compartment at 20°C, where externalization of endocytosed membrane protein is unaffected (Matlin & Simons, 1983). However, the experimental evidence using chloroquine, which neutralizes the acidic endosome compartment, argues both in favour (Salas *et al.*, 1983) and against (Moore *et al.*, 1983) an acidic exocytic pathway, so

the question must therefore be considered open at present.

It is commonly assumed that the cellular compartments can communicate with each other by means of transport vesicles. In order to maintain the membrane specificity of the different compartments, Palade (1982) has suggested that a mechanism of stabilization ensures a transport of the same membraneous micro-domain in both directions of the vesicle flow. This hypothesis implies that a net flow of constituents are transported to a compartment where de-stabilization occurs; in this way the processes of stabilization/destabilization in the different compartments are able to regulate a sorting of components, destined for the various organelles of the cell. In the absence of destabilization in a compartment, the components will be present but not accumulate. Such a model fits well with the presence of small amounts of microvillar enzymes in the compartments that communicate via transport vesicles (the basolateral plasma membrane, the lysosomes). The mechanism of the stabilization/destabilization processes may conceivably occur by means of specific receptors or by a spontaneous aggregation/disaggregation of the proteins that could be regulated by differing environments in the organelles such as changes in pH or Ca^{2+} concentration.

Intracellular processing

N-Linked glycosylation

The idea of the existence of a common intracellular pathway for the transport of enzymes to the microvillar membrane can be extended to predict similarities in the nature of processing of the primary translation products. It is well established that the microvillar enzymes become glycosylated to a high-mannose form (Table 1). This occurs at an early stage in their biogenesis, presumably co-translationally or very shortly thereafter, since non-glycosylated precursors have not been demonstrated in any reported labelling experiments. High-mannose glycosylation is a well characterized processing of secretory and membrane proteins which takes place in the rough endoplasmic reticulum and consists of the transfer *en bloc* of a mannose-rich, branched oligosaccharide from dolicholphosphate to susceptible asparagine residues on the nascent polypeptide (Hubbard & Ivatt, 1981; Hughes & Butters, 1981). Several of these core oligosaccharides are transferred to the polypeptides of the microvillar enzymes; for pig intestinal aminopeptidase N it was estimated that about 10 high-mannose cores per polypeptide are attached (Danielsen *et al.*, 1983b), and for rat kidney γ -glutamyl transpeptidase the number was estimated to 5–6 (Capraro & Hughey, 1983). In the

Table 1. Molecular forms of microvillar enzymes during biosynthesis

 $10^{-3} \times M_r$ of:

Enzyme	Species	Tissue	$10^{-3} \times M_r$ of:			Endoglycosidase H sensitivity	$10^{-3} \times M_r$ of mature form*	Transport time (min.)	References
			Primary translation product/ non-glycosylated form	Transient form	10 ⁻³ × M _r of mature form*				
Amino-peptidase N (EC 3.4.11.2)	Pig	Intestine	115	140	166 (123+62)	+		60-90	Danielsen (1982); Danielsen <i>et al.</i> (1982b, 1983b); Sjöström <i>et al.</i> (1978)
	Rat	Intestine	-	120†	130	Not detected		60-180	Ahnen <i>et al.</i> (1982)
	Rat	Intestine	-	<130	130	+		>180	Ahnen <i>et al.</i> (1983)
	Pig	Kidney	115	140	160	+		90	J. R. Stewart & A. J. Kenny (personal communication)
Amino-peptidase A (EC 3.4.11.7)	Pig	Intestine	-	140	170	+		60-90	Danielsen <i>et al.</i> (1983a)
	Pig	Kidney	-	145	170	+		90	J. R. Stewart & A. J. Kenny (personal communication)
Dipeptidyl peptidase IV (EC 3.4.14.5)	Pig	Intestine	-	115	137	+		60-90	Danielsen <i>et al.</i> (1983a)
	Pig	Kidney	-	115	130	+		90	J. R. Stewart & A. J. Kenny (personal communication)
Endo-peptidase- 24.11 (EC 3.4.24.11) γ-Glutamyl transpeptidase (EC 2.3.2.2)	Pig	Kidney	-	88	93	+		90	J. R. Stewart & A. J. Kenny (personal communication)
	Rat	Kidney	63	78	(51+22)	Not detected		~120	Nash & Tate (1982, 1984)
Sucrase- isomaltase (EC 3.2.1.48-10)	Rat	Kidney	63	78	(50+23)	Not detected		-	Kuno <i>et al.</i> (1983); Matsuda <i>et al.</i> (1983)
	Rat	Kidney	-	75	(49.5+29)	+, -		-	Capraro & Hughey (1983)
	Rabbit	Intestine	240, 270	-	275 (120+140)	Not detected		-	Wacker <i>et al.</i> (1981); Hauri <i>et al.</i> (1979); Brunner <i>et al.</i> (1979)
	Pig	Intestine	225	240	265 (140+150)	+		60-90	Danielsen (1982); Danielsen & Cowell (1984)
Maltase- glucoamylase (EC 3.2.1.20)	Pig	Intestine	200	225	245 (125+135)	+		60-90	Danielsen <i>et al.</i> (1983a); Danielsen & Cowell (1984)
Lactase- phlorizin hydrolase (EC 3.2.1.23-62)	Pig	Intestine	210	225	245 (160)	+		60-90	E. M. Danielsen (unpublished work; Sjöström <i>et al.</i> (1983)

* Values in parentheses indicate the M_r of proteolytically cleaved mature forms.

† This transient form was soluble rather than membrane-bound.

pulse-chase experiments reported, the high-mannose glycosylated form of the microvillar enzymes prevails for 30–60 min. After this period, which presumably represents the time required for traffic from the rough endoplasmic reticulum to the Golgi complex (Hubbard & Ivatt, 1981), the enzymes are converted to a molecular form of the same size as that observed in microvilli, before any further proteolysis. The Golgi complex is known to contain the enzymes responsible for this conversion, a step that soon renders the *N*-linked oligosaccharides resistant to endoglycosidase H. It consists in the removal of mannose residues (trimming), followed by attachment of sugars of complex glycosylation, such as galactose, sialic acid and fucose (Hubbard & Ivatt, 1981). The first four of the enzymes involved in this processing have been localized to the cisternae on the *cis* side of the Golgi complex (Dunphy & Rothman, 1983), whereas the last two transferases were found on the *trans* side (Roth & Berger, 1982), thereby suggesting a *cis-trans* directional movement through the organelle, concomitant with the processing. Despite its sequential character, the pool sizes of the intermediate forms are very small and escape detection in pulse-chase labelling experiments, indicating that the time interval between the different steps of trimming and complex glycosylation of the individual polypeptides is very short. However, by using monensin, a blur of labelled polypeptides, extending below and above the high-mannose glycosylated form of pig intestinal aminopeptidase N, was evidence that the processing was arrested at various incomplete stages (Danielsen *et al.*, 1983c). Ahnen *et al.* (1983), studying the conversion from transient to mature form of rat intestinal aminopeptidase N, concluded, on the basis of membrane separation by two-phase counter-current distribution, that the major steps in this processing occur in the endoplasmic reticulum. This interesting conclusion suggests the existence of a processing pathway different from the one described above, and deserves further investigation.

O-Linked glycosylation

The considerable M_r difference (20000–30000) between the transient and mature forms of several microvillar enzymes suggests that other types of processing than *N*-linked glycosylation might occur. The alkaloid swainsonine (Colegate *et al.*, 1979) is a specific inhibitor of mannosidase II, one of the trimming enzymes mentioned above (Tulsiani *et al.*, 1982) and it causes the formation of 'hybrid' *N*-linked oligosaccharides of about the same size as the high-mannose cores (Tulsiani & Touster, 1983; Gross *et al.*, 1983). They are described as 'hybrid' because they retain sensitivity to endoglycosidase H even though they are com-

plex glycosylated. When pig intestinal explants were organ cultured in the presence of swainsonine, a new molecular form of aminopeptidase N was observed, only marginally smaller than the normal, mature form (160000 versus 166000), but still sensitive to endoglycosidase H (Danielsen *et al.*, 1983d). The considerable increase in M_r above the size of the high-mannose glycosylated form (20000) seen in the presence of swainsonine was tentatively attributed to *O*-linked glycosylation on the following ground. In contrast to the high-mannose glycosylated form, the mature form was susceptible to mild alkaline treatment, indicating the presence of *O*-linked glycosylation on only the latter form. In addition, we have recently observed that the high-mannose and complex glycosylated forms of pig intestinal aminopeptidase N and sucrase-isomaltase after treatment with endoglycosidase F [a glycosidase that cleaves both types of *N*-linked oligosaccharides (Elder & Alexander, 1982)] still migrate as discrete bands rather than coincide (Danielsen & Cowell, 1984). This further indicates that the size difference between the two forms cannot be ascribed solely to processing of *N*-linked carbohydrate. Since the forms of pig intestinal sucrase-isomaltase and dipeptidyl peptidase IV in the presence of swainsonine were also of sizes comparable with the corresponding mature (but not proteolytically cleaved) enzymes, it was suggested that these enzymes also receive *O*-linked glycosylation during their passage through the Golgi complex. For sucrase-isomaltase, there is evidence that residue 11 near the *N*-terminus of the isomaltase subunit of the rabbit enzyme is a glycosylated threonine (Frank *et al.*, 1978). Herscovics *et al.* (1980), studying the labelled glycopeptides of Pronase-digested rat intestinal epithelial cell membranes, suggested that a fraction, containing a heterogeneous mixture of large glycopeptides, might be *O*-glycosidically linked. It can be speculated that this type of processing, known to take place for the E1 protein of the corona virus (Niemann & Klenk, 1981) is a general type of modification in the biogenesis of microvillar enzymes.

Proteolytic cleavage

It is well established that pancreatic proteinases cleave some intestinal microvillar enzymes extracellularly after assembly in the membrane. Thus, both trypsin and chymotrypsin act on pig aminopeptidase N (Sjöström *et al.*, 1978). Hauri *et al.* (1979) found that only elastase was able to cleave the precursor of rat sucrase-isomaltase *in vitro*, suggesting that this proteinase in particular is responsible for this post-translational processing. For pig sucrase-isomaltase (Sjöström *et al.*, 1980) and maltase-glucoamylase (Sørensen *et al.*, 1982), not only elastase but also trypsin and chymotrypsin

cleaves the precursor into polypeptides comparable in size to the 'naturally occurring' subunits. Under physiological conditions, all three proteinases may, to a variable extent, be active in this processing.

In contrast with the other microvillar enzymes studied, γ -glutamyl transpeptidase and lactase-phlorizin hydrolase are subjected to processing by proteolytic cleavage intracellularly. Nash & Tate (1982), studying the biogenesis of rat kidney γ -glutamyl transpeptidase by labelling of cultured kidney slices, observed that the M_r 51 000 and 22 000 subunits of the mature enzyme are derived from an intracellularly located common precursor of M_r 78 000. Since this could be labelled with [^3H]-fucose, it was concluded that the cleavage probably occurs after the arrival in the Golgi complex. Only the subunits were found to appear in the microvillar membrane. In cell-free translation, a non-glycosylated polypeptide of the enzyme of M_r 63 000 could be processed to the M_r 78 000 high-mannose glycosylated form by dog pancreatic microsomes (Nash & Tate, 1984). On further incubation of the translation mixture, the precursor was cleaved, yielding two polypeptides of the size of the subunits. This result suggests that the rough endoplasmic reticulum is at least partially responsible for the proteolytic cleavage. Studying the same enzyme by labelling rats *in vivo*, Capraro & Hughey (1983) similarly found an M_r 75 000 polypeptide to be the precursor of both subunits. The initial susceptibility of all three polypeptides to endoglycosidase H led the authors to suggest that the cleavage of the majority of the M_r 75 000 precursor takes place before reaching the Golgi complex, whereas about 30% is cleaved at a later time. From labelling studies of the rat enzyme *in vivo*, Kuno *et al.* (1983) and Matsuda *et al.* (1983) concluded on the basis of cell fractionation experiments that the conversion of the precursor into the subunits does not occur before reaching the microvillar membrane. Thus, at least three organelles have been proposed as candidates for the proteolytic conversion of the γ -glutamyl transpeptidase precursor. It remains to be established how many of these actually play a role under physiological conditions; given the demonstrated susceptibility of the precursor to proteolytic cleavage, the results obtained must be cautiously interpreted in the light of the possibility of preparational artefacts. Capraro & Hughey (1983) hypothesized that the different cellular sites of cleavage might reflect the existence of different but closely related gene products or alternatively, that incomplete cleavage in the rough endoplasmic reticulum could be caused by the competing reaction of high-mannose glycosylation. Further work is needed to solve this problem. It would also be of interest to establish

which proteinase cleaves the precursor in the microvillar membrane; here endopeptidase-24.11 could be a candidate.

Both human and pig lactase-phlorizin hydrolase, which each in their mature form are composed of two subunits of M_r 160 000, have been found to appear as larger precursors (Skovbjerg *et al.*, 1984; E. M. Danielsen, unpublished work; Sjöström *et al.*, 1983). The pig enzyme appeared as an endoglycosidase H-sensitive polypeptide of M_r 225 000 in pulse-chase labelling experiments. A molecular form of M_r 245 000 and the mature subunit of M_r 160 000, both barely sensitive to endoglycosidase H, appeared after 60 min of chase, but only the latter was expressed in the microvillar membrane, indicative of an intracellular cleavage. The cleavage was inhibited by the presence of leupeptin during labelling, which gave rise to the appearance of the precursor in the microvillar membrane, indicating that proteolysis was not essential for transport. The results suggest that the proteolytic cleavage takes place after trimming and complex glycosylation, i.e. in the distal end of the Golgi complex or possibly as a post-Golgi event. A similar site was proposed for the cleavage of the fowl plague virus haemagglutinin (Matlin & Simons, 1983). Many prohormones undergo a similar proteolytic cleavage late during their intracellular transport, and it has been speculated that a common family of proteinases are responsible for this type of processing (Garoff *et al.*, 1980).

Other types of intracellular processing

Different types of intracellular processing from those described in this section have not been reported for the microvillar enzymes. One such type, acylation, has been found to be active not only for viral but also for some eukaryotic plasma membrane proteins (Schlesinger *et al.*, 1980). However, labelling of pig intestinal explants for 20 h with high concentrations of [^3H]palmitic acid failed to label any microvillar protein components, arguing against acylation occurring as a processing step (Danielsen *et al.*, 1983d). Labelling under similar conditions with [^{32}P]phosphate also failed to label any bands in the microvillar fraction, suggesting that phosphorylation does not take place (E. M. Danielsen, unpublished work).

It has been observed that tunicamycin, an inhibitor of *N*-linked glycosylation (Schwarz & Datema, 1980), led to a drastic decrease in the expression of microvillar proteins (Danielsen *et al.*, 1983b; Danielsen & Cowell, 1984). The presence of leupeptin during labelling was able to restore partially the expression of pig intestinal aminopeptidase N, sucrase-isomaltase and maltase-glucosylase. The resulting non-glycosylated forms were not transported to the microvillar membrane

but persisted intracellularly. These results imply that a leupeptin-sensitive, intracellularly located proteinase recognizes and degrades newly synthesized, malprocessed enzymes. It can be speculated that this degradation system, revealed here by the combined use of tunicamycin and leupeptin, is also physiologically active, in the absence of drug-induced malprocessing, as a type of quality control of newly synthesized proteins destined for insertion into cellular membranes. The effect of tunicamycin, even during pulses as short as 20 min, indicates that the degradative de-routing occurs early in the biosynthetic pathway, probably in the rough endoplasmic reticulum. Such a capacity of the rough endoplasmic reticulum would be compatible with recently proposed models for catabolic regulation of protein expression (Bienkowski, 1983), but a crinophagic pathway, leading to degradation in the lysosomes, is also possible (Blok *et al.*, 1981a,b).

The importance of processing for biological activity

Any study of the relationship between the processing, both glycosylation and proteolysis, and the acquisition of biological activity is evidently hampered by the technical difficulties in obtaining sufficiently large and pure preparations of the transient forms of the microvillar enzymes for determination of specific activity. For pig sucrase-isomaltase and maltase-glucoamylase, it has been shown that the extracellular proteolytic cleavage of the precursors has little, if any, effect on the specific activity (Sjöström *et al.*, 1980; Sørensen *et al.*, 1982), but it is unknown whether the same holds for the intracellular cleavage of γ -glutamyl transpeptidase and lactase-phlorizin hydrolase. The importance of high-mannose glycosylation for biological activity has generally been found to vary considerably for various proteins (Gibson *et al.*, 1980), but the drastic effect of tunicamycin (Danielsen & Cowell, 1984) suggests that it is essential for the correct folding of at least some microvillar enzymes. The significance of the Golgi-associated processing of trimming and complex glycosylation, and probably *O*-linked glycosylation, has been studied for some of the pig intestinal microvillar enzymes (H. Sjöström, O. Norén & E. M. Danielsen, unpublished work). When a Ca^{2+} -precipitated membrane fraction was treated with papain prior to solubilization with Triton X-100 and subsequent purification by immunoabsorbent chromatography, essentially pure forms of high-mannose glycosylated aminopeptidase N, maltase-glucoamylase and sucrase-isomaltase were obtained. The explanation for this is probably that the papain treatment releases the complex glycosylated forms, present either in contaminating microvillar vesicles or in intracellular mem-

branes that have not vesiculated in a way that protects the enzymes from the action of the proteinase. Comparison of enzymic and immunological activity of the high-mannose glycosylated and complex glycosylated (mature) forms showed no difference in specific activity for aminopeptidase N and maltase-glucoamylase, but for sucrase-isomaltase the high-mannose glycosylated form had a markedly lower specific activity. The varying importance of high-mannose glycosylation might therefore also hold true for the glycosylation processing, taking place in the Golgi complex.

Formation of the microvillus

Information regarding the assembly *de novo* of the microvillus is as yet limited. Microvillar stalked-type enzymes are not obligatory components for the formation of the microvillus, as these enzymes are not present in large amounts in the crypt cells (Nordström *et al.*, 1968; Skovbjerg, 1981), although microvilli are seen on the luminal surface of the maturing cells (Shiner, 1983). For aminopeptidase N, regulation of expression is at the transcriptional or translational level, as no synthesis can be detected in crypt cells, with [^{35}S]methionine labelling in the presence of the proteinase inhibitor leupeptin (E. M. Danielsen, unpublished work). Expression of these enzymes thus seems to be turned on late in enterocyte differentiation. This is in contrast to two proteins thought to be obligatory for microvillus formation, a 140 kDa membrane glycoprotein and villin, which have a similar rate of synthesis in undifferentiated and differentiated HT29 cells (Louvard *et al.*, 1983).

The formation of the microvillus might occur by an ordered process of membrane-cytoskeletal interactions, analogous to the start of the budding process described for enveloped viruses (Garoff *et al.*, 1982). The steps for the generation of the microvillus are suggested to be as follows. Microdomains in the apical plasma membrane, corresponding to the tips of the microvillus (dense plaques), are generated, and end-on binding of actin occurs. The components forming the dense plaques have a vinculin-like function, but are not immunologically related to vinculin (Geiger *et al.*, 1981). At least three further processes are necessary for microvillar generation: actin filament elongation, filament bundling, and formation of the filament-membrane side-on interactions. The sequence of events is unknown, but the processes must occur concomitantly to some extent. The formed actin filaments are orientated so that the 'barbed' ends are at the tip of the microvillus (Mooseker *et al.*, 1981). Elongation of the actin filaments occurs *in vitro* at both the 'barbed' and

'pointed' ends (Mooseker *et al.*, 1981), although the side of elongation *in vivo* is unclear. Bundling of the 20 or so actin filaments that are found in each intestinal microvillus probably occurs with the aid of villin, an actin-binding protein, and fimbrin (Matsudaira *et al.*, 1983). The folding of the lipid membrane occurs by cross-linking of the actin filaments to a membrane protein(s) via the 110kDa protein-calmodulin complex (Howe & Mooseker, 1983). A 140kDa protein, recently purified and characterized (Coudrier *et al.*, 1983), is implicated as the membrane protein participating in this folding/microvillar-elongation process.

The mechanism regulating microvillar length is unknown, but elongation may be stopped by exclusion of the membrane protein(s) involved in the membrane-microfilament interaction from the pits between the microvilli; in fact, the 140kDa membrane protein is decreased in or absent from the pits of rat enterocytes (Coudrier *et al.*, 1983). In addition to the major structural and regulatory proteins of the microvillus cytoskeleton, briefly mentioned above, other molecules may 'help' in the assembly process, as demonstrated for the formation of other complex supramolecular structures such as bacteriophage T4 (Wood, 1978).

Once the microvillus is formed, how are the newly synthesized microvillar enzymes inserted and distributed in the brush border membrane? Vesicles containing newly synthesized glycoproteins have been demonstrated in the apical part of the enterocytes (Bennet *et al.*, 1974; Bernadac *et al.*, 1984).

These vesicles may fuse with the plasma membrane forming the pits, and lateral diffusion of the microvillar membrane enzymes then distributes the enzymes, as there is probably no restriction by interaction with the cytoskeleton. Fusion of vesicles along the entire length of the microvillus is unlikely, as the diameter of the transport vesicles (approx. $0.1\mu\text{m}$) is comparable with that of the cytoskeleton-filled microvillus. In contrast to direct microvillar insertion, other routes have also been suggested, as discussed in an earlier section.

In the future, understanding of the assembly of the microvillus might be expected from studies involving cell lines whose differentiation can be controlled (Pinto *et al.*, 1982) and by isolation of the genes that are essential for microvillus formation, followed by transformation of non-polarized cells and by reconstruction of the microvillus structure by isolated components.

Conclusions

To summarize, the data collected in this Review suggest a common theme in the biogenesis of microvillar enzymes from both intestine and kidney (Fig. 2). The *leitmotiv* of this theme is the shared biosynthetic route of transport and the nature of the processing that converts the primary translation products into the mature enzymes. Small variations in the kinetics of transport may well exist which might reflect minor deviations

Fig. 2. Intracellular transport and processing of microvillar enzymes

The intracellular route of transport of newly synthesized microvillar enzymes (1–4) and the molecular forms associated with the successive steps of processing (exemplified by aminopeptidase N). (1) Synthesis of the primary translation product of M_r 115,000 in the rough endoplasmic reticulum where co-translational high-mannose glycosylation to yield the M_r 140,000 form also occurs. (2) Intermediate steps in the Golgi complex between the transient and mature forms. (3) The mature M_r 166,000 form in the post-Golgi transport to the microvillar membrane. (4) Extracellular cleavage by pancreatic proteinases of the M_r 166,000 form to yield the polypeptides of M_r 123,000 and 61,000 (A- and B-subunits). The left side of the Figure shows the various inhibitors that have been observed to interfere at different stages in the transport or processing of microvillar enzymes.

from a common pathway. Likewise, the fine details of the co- and post-translational glycosylation are bound to vary quantitatively among the enzymes and also for the same enzyme, synthesized by either the intestine or kidney (Norén *et al.*, 1979; Fulcher *et al.*, 1983). Differences that might be of a more profound significance are the intracellular cleavages of the precursors of γ -glutamyl transpeptidase and lactase-phlorizin hydrolase.

Despite the considerable interest that has focused on this subject in the recent years, some important problems remain unsolved. One of these is to establish beyond doubt if the signal peptides of these enzymes remain uncleaved. Another problem is to resolve the questioned role of the basolateral plasma membrane in the transport. This problem may well be linked to the more general one of sorting, which currently attracts the attention also of cell biologists working in related fields.

Work devoted to the biosynthesis of microvillar enzymes has so far, by using specific antibodies as probes, been concerned with translational and post-translational events. By employing similarly specific DNA probes, it should be possible in the future to extend the subject to include also events at earlier stages in the expression of these enzymes. The differently sized primary translation products of the microvillar enzymes and their individual regulation in the intestine during embryonic development may be a promise of more individuality of these enzymes as far as their gene organization and regulation are concerned. Here, inspiration may come from other eukaryotic plasma membrane proteins like the H-2 K^d antigen (Kvist *et al.*, 1983), where the coding sequences for the cytoplasmic domain, the membrane spanning segment, the three extracellular domains and the signal sequence were found on separate exons on the gene.

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