Biosynthesis of intestinal microvillar proteins

Rapid expression of cytoskeletal components in microvilli of pig small intestinal mucosal explants

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Using alkaline extraction to separate cytoskeletal and membrane proteins of intestinal microvilli, the kinetics of assembly of these two microvillar protein compartments was studied by pulse-chase labelling of pig small intestinal mucosal explants, kept in organ culture. Following a 10 min pulse of [^{35}S]methionine, the membrane proteins did not appear in the microvillar fraction until after 40–60 min of chase. In contrast, the cytoskeletal components, of which the 110-kDa protein and villin were immunologically identified, were expressed in the microvillar fraction immediately after the 10 min pulse. These different kinetics of appearance indicate that the two microvillar protein compartments have separate mechanisms of biosynthesis and microvillar expression.

Microvillus *Biosynthesis* *Cytoskeleton* *Membrane protein* *Small intestine*

1. INTRODUCTION

Intestinal microvilli contain two protein compartments; one being the membrane proteins of which the most abundant are peptidases or glycosidases (review [1]), the other being constituted by the components of the cytoskeleton, responsible for the microvillar structure [2]. A substantial body of information is now available on the molecular organization of microvilli. The central core is composed of a bundle of polarized actin filaments, originating at the tips of the microvilli [3]. Lateral bridges between actin filaments and the microvillar membrane are composed of the 110-kDa protein [4]. Villin acts as a gelation factor, with the ability to bundle or restrict F-actin to short fragments, depending on the Ca^{2+} concentration [5] and calmodulin interacts with several of the core proteins [6].

The biosynthesis of several microvillar enzymes has been studied (review [7]). They are synthesized by ribosomes, attached to the rough endoplasmic reticulum and transported to the microvillar membrane in a membrane-bound state, passing through the Golgi complex en route, where post-translational modifications, yielding the mature enzymes, occur. The uniform kinetics of transport and processing for various enzymes suggest the existence of a common pathway for this type of microvillar protein. In contrast, little is known about the biosynthesis and microvillar expression of the cytoskeletal proteins. In a pulse-chase labelling study on the turnover of microvillar cytoskeletal proteins in chickens in vivo, it was proposed that these components appear more slowly in microvilli than membrane glycoproteins [8]. However, since the chase periods were 6–24 h, it could well be that the kinetics of intracellular transport and microvillar expression of newly synthesized proteins escaped detection in these experiments. This was investigated here, using pulse-chase labelling of intestinal organ cultured

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mucosal explants, a system in which events of processing and transport of newly synthesized proteins can be monitored [7].

2. EXPERIMENTAL

2.1. *Materials*

The sources of materials used for organ culture were as in [9]. Antibodies, raised in rabbits against pig 110-kDa protein and villin, were kindly given by Dr D. Louvard, Institut Pasteur, Paris.

Pig small intestines were kindly supplied by the Department of Experimental Pathology, Rigshospitalet, Copenhagen.

2.2. *Labelling of mucosal explants*

Organ culture [10] of small intestinal explants was performed as in [9] with the culture medium containing 100–200 μCi/ml [^{35}S]methionine. In pulse-chase experiments, the chase medium contained an increased concentration of non-radioactive methionine (2 mM). Explants were kept frozen at −80°C prior to further processing.

2.3. *Fractionation of labelled explants*

All procedures were carried out at 4°C. Aprotinin (2.8 μg/ml) and PMSF (0.1 mM) were present in all buffers.

Cultured explants were thawed, and a microvillar fraction prepared by micropurification as in [11], using $MgCl_2$ as precipitant of intracellular and basolateral membranes, rather than $CaCl_2$. The pelleted microvillar fraction was rinsed twice with 0.3 ml of 50 mM Tris–HCl (pH 7.4).

2.4. *Preparation of microvilli*

A microvillar fraction from frozen, everted pig small intestine was prepared as in [12], using $MgCl_2$ as precipitant.

2.5. *Alkaline treatment of microvilli*

Alkaline treatment [13] of microvilli was performed as follows: microvilli (up to 1 mg protein) were resuspended in 2 ml of 100 mM Na_2CO_3 (pH 11.0) and left for 30 min at 4°C, before centrifugation at 148000 × g for 1 h. The pellets were collected and the supernatants precipitated with an equal volume of 20% trichloroacetic acid, followed by centrifugation at 27000 × g for 10 min, to collect the precipitated protein.

2.6. *Other methods*

SDS–10% polyacrylamide gel electrophoresis (SDS–PAGE) was performed as in [14] and fluorography of the gels according to [15]. Immunoblotting was carried out as in [16] and determination of protein concentration and enzyme activities as in [17].

3. RESULTS

3.1. *Alkaline treatment of microvilli releases cytoskeletal proteins*

Alkaline treatment [13] is a rapid and convenient method for converting closed vesicles to open membrane sheets, thereby releasing the vesicular contents on non-integral membrane proteins. This method has been used to study membranes of rough and smooth endoplasmic reticulum, peroxisomes and mitochondria [18]. Here, alkaline treatment was used to release the cytoskeletal proteins, entrapped as microvilli vesiculate during tissue homogenization, from the microvillar membrane, containing the membrane proteins. Whereas the activities of membrane-bound enzymes [aminopeptidase N (EC 3.4.11.2) and dipeptidyl peptidase IV (EC 3.4.14.5)] could be partially recovered in the membrane fraction after the alkaline treatment (about 40%), they were totally absent in the supernatant. The exclusive presence in the membrane fraction of major bands of M_r 240000 and a blur of bands of M_r 120000–150000, representing maltase–glucoamylase (EC 3.2.1.20), sucrase–isomaltase (EC 3.2.1.48-10) and aminopeptidase N [17,19,20] confirmed this finding (fig.1A). In contrast, the major bands, released by the alkaline treatment, were of lower M_r. Two of these, of 105 kDa (partially released) and 95 kDa, specifically reacted with antibodies to the 110-kDa protein and villin, respectively, thus identifying these proteins (fig.2). The less than complete release of 110-kDa protein is suggestive of a partial attachment to the membrane and agrees with the finding that it acts as a bridge protein between the core filament bundle and the membrane [4]. Actin (M_r 40000), the most abundant protein component in the enterocyte, also predominantly partitioned into the soluble fraction, as did some less abundant polypeptides of M_r 85000, 70000, 32000 and 28000. The 70-kDa polypeptide may be tentatively identified as fimbrin [21], and that of 32 kDa

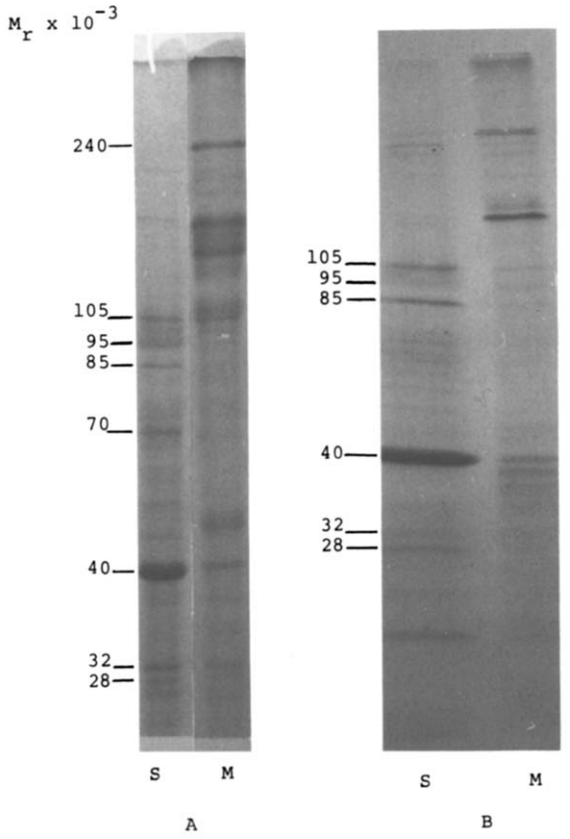

Fig.1. Alkaline treatment of microvilli. Microvilli, obtained either from non-radioactive tissue (A) or from mucosal explants, labelled for 30 min with 100 μCi [^{35}S]methionine (B), were treated with Na_2CO_3 as described in section 2. The soluble (S) and membrane-bound (M) fractions were subjected to SDS–PAGE. After electrophoresis, the gels were stained for protein with Coomassie brilliant blue (A) or prepared for fluorography (B). Apparent M_r values ($\times 10^{-3}$) are shown.

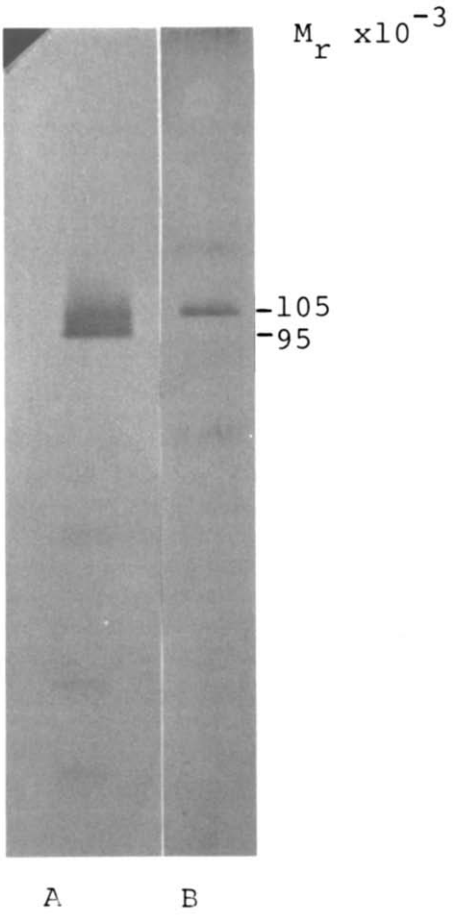

Fig.2. Immunoblotting of the alkaline-released, cytoskeletal proteins. Microvilli, prepared from non-radioactive tissue, were treated with Na_2CO_3 as described in section 2. Protein (20 μg) from the soluble fraction was subjected to SDS–PAGE. The gel was then immunoblotted, using either anti-pig villin serum (A) or anti-pig 110-kDa protein antiserum (B) in dilutions of 1:200 and 1:400, respectively. Apparent M_r values ($\times 10^{-3}$) are shown.

might represent a recently described cytoskeletal component [22]. The conspicuous band of 85 kDa is not a degradation product of either the 110-kDa protein or villin since it did not react with the antibodies in the experiment (fig.2), neither is it likely to be a contaminant since it is also present in ultrapure preparations of microvilli [23]. It could possibly represent an 80-kDa protein which was recently discovered in the cytoskeleton of chicken microvilli [24]. Bands of M_r 220000 and 140000 are likely to be contaminants from the Mg^{2+}-precipitated membrane fraction of which these are very abundant components (not shown).

Alkaline extraction, being a reliable method of separating membrane-bound and cytoskeletal proteins of microvilli, was applied in experiments with labelled intestinal explants. Fig.1B shows the labelling pattern of the two fractions of explant microvilli. The pattern of proteins, released by the alkaline treatment, resembles that obtained for non-radioactive tissue. Thus, this fraction contained the predominant amounts of the 110-kDa

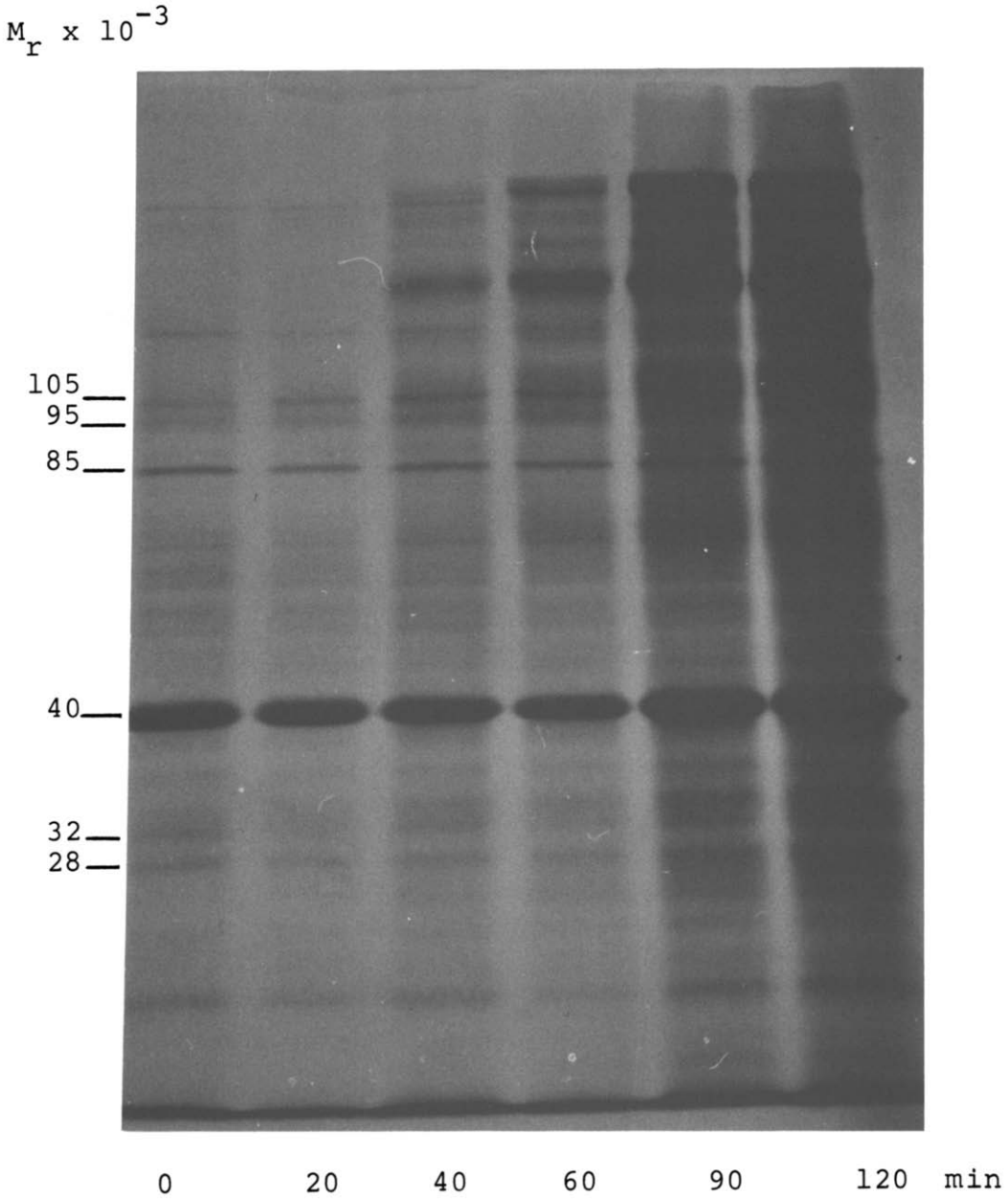

Fig.3. Pulse-chase labelling of microvillar proteins. Mucosal explants were labelled for 10 min with 200 μCi [^{35}S]methionine and chased with non-radioactive methionine for the indicated periods of time (min). Microvillar fractions were prepared and subjected to SDS–PAGE, after which the gel was prepared for fluorography. Apparent M_r values ($\times 10^{-3}$) are shown.

protein and villin and actin. Polypeptides of M_r 85000, 32000 and 28000 were also present in the soluble fraction after alkaline treatment. (The 70-kDa band could be seen as well but appeared less intense than the corresponding non-radioactive polypeptide.) The membrane-bound polypeptides exhibited a different pattern. Pancreatic proteinases are known to cleave the most

abundant microvillar enzymes in vivo [17,19,20]. Since labelling in organ culture was performed in the absence of pancreatic proteinases, the two lanes of membrane-bound polypeptides in fig.1 cannot be directly compared.

3.2. *Pulse-chase labelling of microvillar proteins*

Fig.3 shows a pulse-chase labelling of the microvillar fraction of intestinal explants. The polypeptides, detectable immediately after the 10 min pulse, were found to correspond well with those of the cytoskeletal fraction of microvilli after the alkaline treatment: the 110-kDa protein, villin, actin and polypeptides of M_r 85000, 70000 (faint), 32000 and 28000 could be seen. After 40–60 min chase, other components, especially in the high-M_r region, appeared with increasing intensity. Some of these (M_r 265000, 245000 and 166000) were of similar size to sucrase–isomaltase, maltase–glucoamylase and aminopeptidase N, respectively, and are likely to represent these enzymes which constitute a considerable amount of total microvillar protein mass. In contrast to the cytoskeletal components, these late-appearing polypeptides of high M_r were labelled by [^{3}H]fucose during organ culture for 20 h, indicating that they alone are glycoproteins (not shown).

4. DISCUSSION

Our results clearly indicate that newly synthesized protein components of microvilli reach their destination via two kinetically different routes. One of these has been characterized [7] and is used by the enterocyte to transport and process newly synthesized enzymes from their site of synthesis, the rough endoplasmic reticulum, via the Golgi complex to the microvillar membrane. This membrane-bound pathway requires about 60 min preceding the microvillar expression as demonstrated by the slowly appearing polypeptides in fig.3. In contrast, the labelling pattern, seen immediately after the 10 min pulse, must represent components that reach the microvilli by another route of transport. In fact, it was not possible to detect a lag period in the microvillar appearance of these, even with pulse periods as short as 2 min (not shown). This, together with the observation that none of the rapidly appearing polypeptides were labelled by [^{3}H]fucose, makes it reasonable to propose that these are synthesized by free ribosomes and reach the microvilli by an unknown mode, possibly by diffusion or conceivably by a facilitated mechanism, for instance, by interaction with microtubules. The pattern of rapidly expressed polypeptides was found to correspond well with that of microvillar skeletal components, obtained by alkaline treatment. Of particular interest, there was no evidence of any integral membrane proteins being rapidly expressed in the microvillar membrane. This seems to exclude the possibility of any membrane proteins being directly inserted into microvilli by a 'membrane trigger' mechanism [25].

It has long been known that intestinal microvillar glycoproteins have a rapid turnover [26]. In a recent investigation of the microvillar cytoskeletal proteins, it was demonstrated that although these proteins have a relatively slow turnover compared to the membrane proteins, the cytoskeleton once formed is not a static entity but undergoes continuous renewal [8]. The finding of different turnover rates for the cytoskeletal and membrane protein compartments of the microvilli is parallelled by the observation that the two types of components are expressed in microvilli by different mechanisms. It is evident that a high degree of coordination of these events must exist to maintain the highly ordered structure of the brush border. How this is achieved is an intriguing problem that still remains to be solved.

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