Biosynthesis of intestinal microvillar proteins

Surface expression of aminopeptidase N is not affected by chloroquine

E. Michael Danielsen, Hans Sjöström and Ove Norén

Department of Biochemistry C, The Panum Institute, University of Copenhagen, 3 Blegdamsvej, DK-2200 Copenhagen N, Denmark

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The effect of chloroquine on the biosynthesis of pig intestinal aminopeptidase N (EC 3.4.11.2) was studied by labelling with [^{35}S]methionine in organ cultured mucosal explants. The lysosomotropic agent did not alter the molecular size of either the transient or the mature form of the enzyme and did not markedly influence the relative intracellular distribution of the two forms. The microvillar expression of aminopeptidase N during labelling periods of 80–120 min was found to be unaffected by chloroquine. Together these data indicate that pH neutralization of the acidic compartments of the cell bears no consequence on the intracellular transport of the newly synthesized microvillar enzyme. This suggests that the acidic compartments are not involved in the post-Golgi transport and that this, in turn, probably occurs via a constitutive rather than a regulated pathway.

Microvillar membrane *Biosynthesis* *Intracellular transport* *Chloroquine* *Aminopeptidase N* *Organ culture*

1. INTRODUCTION

Endocytosis and exocytosis are well known mechanisms whereby eukaryotic cells import and export proteins [1]. The vesicular pathway responsible for internalization of molecules and particles from the cell surface leads to the secondary lysosomes, but radioautographic evidence has demonstrated also a considerable membrane traffic from the plasmalemma to the *trans* side of the Golgi complex [2]. The vesicular pathway used by the cell to externalize newly synthesized proteins, destined for export, leads from the rough endoplasmic reticulum, via the Golgi complex, to the plasma membrane [3]. An unsolved problem in this context is to what extent the exo- and endocytic pathways are interconnected. Evidence favouring the existence of a link between endo- and exocytosis is the finding that apical aminopeptidase N (EC 3.4.11.2) in MDCK cells, when cross-linked by an antibody and subsequently endocytosed, rapidly reappeared in the plasma membrane at the site of cell-cell contact [4].

It has been shown that monensin inhibits the expression in the microvillar membrane of newly synthesized enzymes [5]. A concomitantly occurring vacuolization of the Golgi complex and inhibition of complex glycosylation supported the interpretation that the intracellular transport was arrested in this organelle. However, monensin has also been reported to inhibit Semliki Forest virus penetration into cultured cells [6] and delivery of absorbed ligands to secondary lysosomes [7], probably by increasing the pH in the endocytic vacuoles (endosomes) [8]. It therefore cannot be excluded that the transport inhibition by monensin, observed in [5], occurs at a post-Golgi stage nor that acidic endosomes are involved in the final transfer of enzymes to the microvillar membrane.

Lysosomotropic agents such as chloroquine are known to interfere with the endocytic pathway [9] and have been shown to cause an enlargement of

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dense bodies in the apical region of absorptive cells of organ cultured human small intestinal mucosa [10]. To study the possible involvement of acidic endosomes in microvillar enzyme biosynthesis, the effect of chloroquine on the surface expression of aminopeptidase N was tested in organ cultured pig small intestine.

2. EXPERIMENTAL

2.1. *Materials*

Equipment for performing organ culture, including Trowell's T-8 medium, foetal calf serum, sterile plastic dishes with grids and [^{35}S]methionine (spec. act. > 1000 Ci/mmol), were obtained as in [11]. Chloroquine was a product of Sigma, St. Louis, USA.

Pig small intestines were kindly given by the department of Experimental Pathology, Rigshospitalet, Copenhagen.

2.2. *Methods*

Organ culture of intestinal mucosal explants was performed as in [11]. In experiments with chloroquine, the drug was added to the culture medium (10^{-4} M) 1 h prior to continuous labelling with [^{35}S]methionine (50–100 μCi/ml) for 80–120 min (still in the presence of chloroquine). Control explants without the addition of chloroquine were incubated in parallel. The labelled explants were fractionated as in [12] with the modification that $MgCl_2$ was substituted for $CaCl_2$ in the precipitation of intracellular and basolateral membranes. Microvillar enzymes were immunopurified by line immunoelectrophoresis against specific antibodies [13] and the purified enzymes subjected to SDS-polyacrylamide gel electrophoresis as in [14]. Fluorography of the gels was carried out as in [15]. Developed films were scanned in a Kipp & Zonen DD2 gel scanner (Delft, The Netherlands).

3. RESULTS AND DISCUSSION

In previous work, the explant system has proved suitable for studying the effect on microvillar protein biosynthesis of drugs like monensin, colchicine, swainsonine and tunicamycin [5,16,17]. The divalent cation precipitation technique offers a rapid and efficient separation of microvillar and intracellular (and basolateral) membranes, and the relative amounts of radioactive (newly synthesized) microvillar enzyme in the two fractions is a measure of the explants' ability to transport the enzyme to the cell surface.

Fig.1 shows the gel electrophoretic pattern of aminopeptidase N from controls and chloroquine-exposed explants. In the controls, both a transient, high mannose glycosylated polypeptide of M_r 140000 and the mature, complex glycosylated form of M_r 166000 were present in the Mg^{2+}-precipitated membrane fraction whereas only the latter form was detectable in the microvillar fraction. The presence of chloroquine did not markedly influence the labelling of the enzyme and the distribution of transient and mature forms was qualitatively similar to the control pattern. The quantitative distribution of labelled aminopeptidase N between the two membrane fractions, based on densitometric scans of moderately exposed fluorographs, is listed in table 1. (Enzymes from corresponding control and chloroquine-exposed explants were aligned on the same fluorograph.) For the controls, an increasing proportion of the total amount of labelled aminopeptidase N appeared in the microvillar membrane during continuous labelling from 80 to 120 min, consistent with the finding that it takes 60–90 min for a newly synthesized protein to reach the microvillar membrane [12]. The presence of chloroquine did not alter markedly the distribution of aminopep-

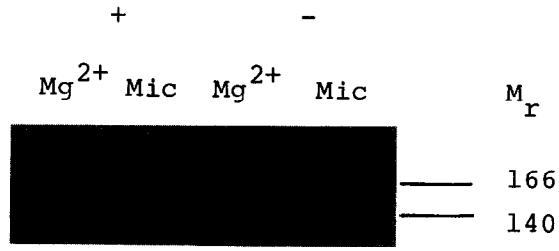

Fig.1. Effect of chloroquine on transient and mature forms of aminopeptidase N. Explants were labelled for 90 min with [^{35}S]methionine (100 μCi/ml) in the absence (−) or presence (+) of chloroquine and fractionated into a Mg^{2+}-precipitated membrane fraction (Mg^{2+}) and a microvillar fraction (Mic). Aminopeptidase N was immunopurified from the solubilized membrane fractions and subjected to SDS-polyacrylamide gel electrophoresis. After electrophoresis, the gel was prepared for fluorography. Exposure time: 10 days. Apparent M_r values ($\times 10^{-3}$) are shown.

Table 1

Effect of chloroquine on microvillar expression of aminopeptidase N

Explants	Distribution of labelled aminopeptidase N (%)	
	Mg^{2+}-precipitated membrane fraction	Microvillar fraction
80 min, −	64	36
80 min, +	72	28
100 min, −	58	42
100 min, +	61	39
120 min, −	21	79
120 min, +	18	82

Explants were labelled continuously with 100 μCi/ml [^{35}S]methionine for 80, 100 and 120 min in the absence (−) or presence (+) of chloroquine. Aminopeptidase N, purified from the Mg^{2+}-precipitated membrane fraction and the microvillar fraction was subjected to SDS-polyacrylamide gel electrophoresis, followed by fluorography. The relative amounts of labelled enzyme in corresponding fractions was determined by densitometry

tidase N at any of the labelling periods, indicating that the drug has very little if any effect on the surface expression of the enzyme.

In table 2 is shown the relative intracellular distribution between the transient form of M_r 140000 and the mature form of M_r 166000. In the controls, the pool size of the former is 2–3 times that of the complex form, indicating that a newly synthesized protein spends more time in the transport from the rough endoplasmic reticulum to the Golgi complex than from here to the microvillar membrane. Within the accuracy of the experiment, chloroquine was not found to influence this distribution, suggesting that the pool sizes of the transient and mature forms and the membrane traffic they represent are not affected by the drug.

The possibility of the microvillar fraction containing membranes of intracellular, for instance endosomal, origin was tested by studying the accessibility of aminopeptidase N to treatment with trypsin. Both the 140-kDa and 166-kDa polypeptides of the Mg^{2+}-precipitated membrane fraction were protected from the proteinase in the absence of Triton X-100, consistent with the notion of an intraluminal location of aminopeptidase N in intracellular membranes (fig.2). In contrast, aminopeptidase N from the microvillar fraction was highly sensitive to trypsin; the treatment degraded 85% of the 166-kDa polypeptide from controls and 87% from chloroquine-treated explants, respectively (as determined by densitometric scans of the fluorographs). Since degradation of enzyme from the latter explants was no less than from the controls, it rules out the possibility of endosomes, present in the microvillar fraction, harbouring the labelled, newly synthesized aminopeptidase N.

The result of this work indicates that pH neutralization of the acidic compartments of the cell by chloroquine has little if any effect on the intracellular transport and molecular processing of newly synthesized aminopeptidase N and therefore suggests that these compartments are not involved in the post-Golgi transport to the microvillar membrane. Otherwise, an inhibition of transport by the lysosomotropic agent would have been expected, as was observed for lysosomal proteins [18], viral membrane proteins [19] and peptide hormones [20]. Authors in [20] found that ACTH was prevented by chloroquine from reaching the secretory granules by a regulated pathway and in-

Table 2

Effect of chloroquine on intracellular distribution between transient and mature forms of aminopeptidase N

Explants	Distribution of labelled aminopeptidase N (%)	
	High mannose form	Complex form
80 min, −	69	31
80 min, +	76	24
100 min, −	63	37
100 min, +	73	27
120 min, −	83	17
120 min, +	74	26

For experimental details, see legend to table 1. The relative amounts of labelled enzyme in the polypeptides of M_r 140000 (high mannose form) and M_r 166000 (complex form) from Mg^{2+}-precipitated membrane fractions of control (−) and chloroquine-exposed (+) explants were determined by densitometry

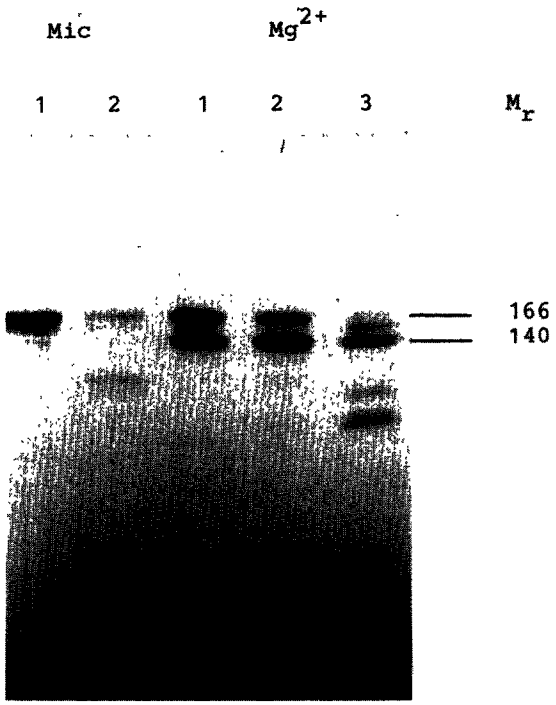

Fig.2. Limited proteolysis with trypsin. Explants were labelled for 80 min with [^{35}S]methionine (100 μCi/ml) in the presence of chloroquine and fractionated into a Mg^{2+}-precipitated membrane fraction (Mg^{2+}) and a microvillar fraction (Mic). Both fractions were incubated with trypsin (0.2 mg/ml) for 45 min at 20°C before the addition of aprotinin (2.8 mg/ml) and cooling on ice. Controls without the addition of proteinase were incubated in parallel. Aminopeptidase N was immunopurified from the solubilized membrane fractions and subjected to SDS-polyacrylamide gel electrophoresis. After electrophoresis, the gels were prepared for fluorography. Exposure time: 4 days. Lane 1, control; lane 2, trypsin-treated; lane 3, trypsin-treated in the presence of Triton X-100. (A similar result was obtained with explants, cultured in the absence of chloroquine.)

stead diverted to a constitutive, chloroquine resistant pathway, leading to immediate secretion from the cells [21]. It seems reasonable to propose that a similar constitutive route is responsible for the insertion of aminopeptidase N in the microvillar membrane. This would be in agreement with the finding that the biosynthesis of microvillar enzymes seems to occur continuously and in the absence of any secretagogue [11,12]. It may therefore well be that the pathway taken by endocytosed aminopeptidase N in MDCK cells to reappear at the cell surface [4] differs from the one used by de novo synthesized enzyme to reach the microvillar membrane.

Like chloroquine, NH_4Cl (10 mM) also failed to affect measurably the surface expression of aminopeptidase N (not shown). Two other microvillar enzymes, sucrase-isomaltase (EC 3.2.1.48-10) and maltase-glucoamylase (EC 3.2.1.20), were studied in parallel with aminopeptidase N. Their biosynthesis was similarly unaffected by chloroquine and NH_4Cl. This further supports the idea of a constitutive pathway being active in the surface expression of microvillar enzymes.

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