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The apical sorting signal on human aminopeptidase N is not located in the stalk but in the catalytic head group

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Human aminopeptidase N carries an apical sorting signal on its ectodomain necessary for its correct transport to the apical membrane in Madin-Darby canine kidney cells. To determine whether the apical sorting signal is localized in the serine/threonine rich stalk or in the catalytic head group, anchor/stalk-minus aminopeptidase N, consisting of the hemagglutinin signal peptide and the catalytic head group of human aminopeptidase N, was expressed in MDCK cells. Anchor/stalk-minus aminopeptidase N was secreted mainly to the apical side. The catalytic head group of human aminopeptidase N thus carries an apical sorting signal.

Aminopeptidase N; Sorting signal; MDCK cells

1. INTRODUCTION

MDCK cells transport newly synthesized apical and basolateral plasma membrane proteins directly from the trans Golgi network to the appropriate membrane domain [1,2]. Until recently it was unclear whether transport to both membrane domains requires sorting signals or if sorting to one of the domains occurs by default and the other one is signal mediated (for a review see [3]). It now seems that both pathways are signal-mediated. It has been known for some time that glycosyl-phosphatidylinositol anchored proteins are sorted to the apical membrane [4,5]. Also a basolateral sorting signal on the polymeric immunoglobulin receptor was identified as a 14-amino-acids cytoplasmic sequence [6]. As yet no sorting signal for non-glycosyl-phospohatidylinositol anchored apical membrane proteins has been identified, but we and others have recently shown that such an apical sorting signal exists [7,8].

In order to identify the apical sorting signal, we have begun a series of studies using the enzyme APN as a model. Human APN is a 967 amino acid protein [9], which is identical to the surface antigen CD 13 [10] and also function as a viral receptor [11]. It was shown that human APN is sorted to the apical membrane when expressed in MDCK cells [12] and recently that the apical sorting signal is localized in the ectodomain of APN [7]. The ectodomain of APN consists of a stalk rich in serine and threonine residues [9] and a large catalytic head group [13]. It may be speculated that a putative sorting receptor, presumably a membrane-spanning protein would interact with molecules to be sorted in a short distance from the membrane e.g. in the stalk. The similar serine/threonine-rich stretch of amino acids seen in some apically expressed proteins e.g. APN [9] and pro-sucrase-isomaltase [14] may thus form a putative apical sorting signal. In the present study we have investigated the role of the serine/threonine rich stalk (amino acid 34–64) for the sorting of APN to the apical domain of MDCK cells. The catalytic head group expressed without the stalk was found to be sorted to the apical domain as efficiently as the wild type APN, which means that a sorting signal is located in the catalytic head group.

Abbreviations: APN, aminopeptidase N; MDCK, Madin-Darby canine kidney; LDL, low density lipoprotein.

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2. MATERIALS AND METHODS

2.1. *Plasmid construction*

Standard techniques were used for DNA manipulations [15]. A cDNA was constructed coding for amino acid 1–17 of hemagglutinin, Ile-Arg-Gly-Ser and amino acid 65–967 of human APN (anchor/stalk-minus APN) using PCR technology. The cDNA was ligated into the pTEJ-4 [16] vector using the *Bam*HI site and transfected into MDCK-cells. The construction of the anchor-minus cDNA has previously been described [7]. In transfections using the pTEJ-4 vector, 1/10 of the transfection DNA was replaced by the pSV2-neo plasmid [17]. Transfections and cell culture were performed as previously described [7].

2.2. *Antibodies*

Two antisera (705 and 706, [18]) both raised in rabbits against human intestinal microvillar membrane proteins were used. Both antibodies were isolated using protein A-Sepharose and further purified by adsorption to dog kidney cytosol proteins immobilized on a Sepharose 4B-resin and to dog kidney microsomes [7,12].

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2.3. *Immunoblotting*

Immunoblot analysis of media and cell extracts were performed as previously described [7,12] using antibody 706. The MDCK-cells were grown to confluence and extracted in 0.1 ml per cm^2 cell culture of electrophoresis sample buffer containing dithiothreitol. 24 h cell culture media were collected and concentrated using centrifugal filtration (Millipore, Ultrafree-MC with a 30,000 NMWL polysulfone membrane) and rediluted to the original volume in dithiothreitol-containing electrophoresis sample buffer. 100 μl samples was applied to the gel.

2.4. *Immunoprecipitations*

Antibody 705 or 706 were allowed to react separately with an equal volume of protein A-Sepharose (50% suspension) overnight at 4°C. The protein A-Sepharose-antibody complex was then washed 3 times in phosphate-buffered saline. Cell culture medium was spun 500 × g for 5 min and the supernatant was allowed to react with an equal volume of protein A-Sepharose-antibody complex overnight at 4°C. The sample was centrifuged for 5 min at 20,000 × g and the supernatant was assayed for aminopeptidase activity.

2.5. *Aminopeptidase assay*

Aminopeptidase activity was measured as earlier described for microvillus aminopeptidase [19] using the substrate L-alanine-*p*-nitroanilide. All experimental figures are given as mean ± 1 S.D.

2.6. *Assay for apical and basolateral delivery of mutant APN*

10^6 cells per well were seeded into 24-mm Transwell chambers (Costar Europe Ltd, Badhoevedorp, The Netherlands). The medium was changed daily. Tightness of filter-grown monolayers was assayed by filling the inner chamber to the brim and allowing it to equilibrate overnight [20]. On day 3 the medium was changed 3 times followed by a 6–24 h incubation with fresh medium. The apical and basolateral media were collected and assayed for aminopeptidase activity.

3. RESULTS

3.1. *Enzymatically active anchor/stalk-minus APN can be expressed in MDCK-cells*

The anchor/stalk-minus APN cDNA coding for amino acids 1–17 of the hemagglutinin signal peptide including the signal peptidase cleavage site, a linker sequence and amino acid 65–967 of APN (Fig. 1) was incorporated into the eukaryotic expression vector, pTEJ-4, and transfected into MDCK-cells. Nontransfected MDCK-cells release no or little aminopeptidase activity into the medium (maximally 3 milliunits/well/24 h) [7]. Two stable MDCK transfectants, Hyb7.2 and Hyb7.4, that express anchor/stalk-minus APN were identified by aminopeptidase activity assay of the media. The Hyb7.2 secreted 55 milliunits/well/24 h and Hyb7.4 secreted 52 milliunits/well/24 h.

In immunoblotting analysis (Fig. 2) a 150 kDa band was clearly visible in the media from Hyb7.2 (lane 4) and Hyb7.4 (lane 6). Immunoreactivity was barely detectable in the corresponding cell extracts (lanes 3 and 5). In MDCK cells transfected with the cDNA coding for anchor-minus APN (Hyb4.3) [7], immunoreactivity was detected in the medium (lane 2) but was barely visible in the corresponding cell extract (lane 1). No immunoreactivity was observed in cell extract or medium from nontransfected MDCK cells (lanes 7 and 8). Corresponding amounts of MDCK-cells expressing the wild type human aminopeptidase N has a clear band of immunoreactivity in the cell extract but no immunoreactivity in the media [7].

Amino acids 1–64 of APN deleted in this study contains one potential Asn-X-Ser/Thr *N*-glycosylation site and many potential *O*-glycosylation sites. It has previously been shown that APN expressed in pig intestinal explants contains significant amounts of both *N*- and *O*-linked carbohydrate [21]. The comparison of the migration ratios of anchor/stalk-minus APN and the anchor-minus APN does not indicate that the stalk is contributing significantly to the size of the ectodomain and therefore is probably not heavily glycosylated despite its many potential *O*-linked glycosylation sites.

3.2. *The anchor/stalk-minus APN is predominantly secreted to the apical side of MDCK cells*

To measure the amount of recombinant protein that is secreted at the apical and basolateral domains of the MDCK-cells, transfectants were grown on polycarbonate filters and 24 h media were collected and assayed for aminopeptidase activity. The anchor/stalk-minus APN

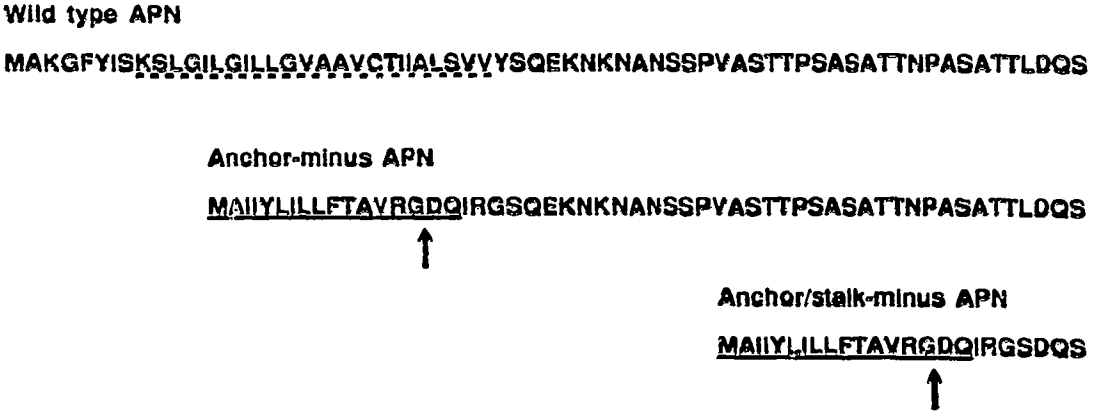

Fig. 1. The N-terminal sequence of the wild-type APN, anchor-minus APN and the anchor/stalk-minus APN. The wild-type APN transmembrane-spanning part is underlined by a dotted line and the hemagglutinin signal peptide is underlined by a solid line. The cleavage site is marked by an arrow.

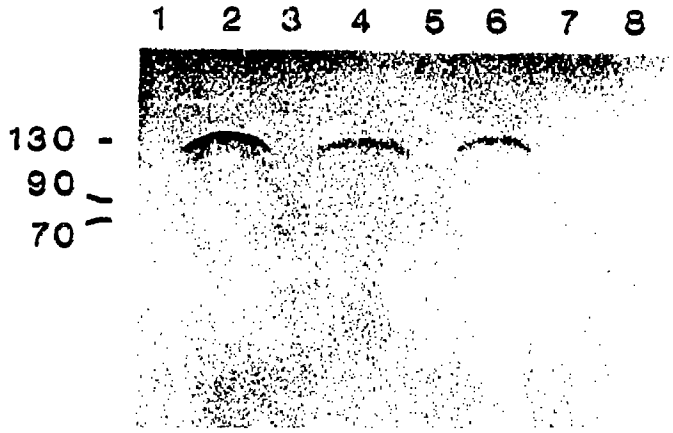

Fig. 2. Expression of anchor/stalk-minus APN in MDCK-cells. Cell extract (lane 1) and medium (lane 2) from the Hyb4.3 clone, cell extract (lane 3) and medium (lane 4) from the Hyb7.2 clone, cell extract (lane 5) and medium (lane 6) from the Hyb7.4 clone and cell extract (lane 7) and medium (lane 8) from nontransfected MDCK-cells were subjected to immunoblot analysis. Approximately equal amounts of cell extracts and media respectively were applied to the polyacrylamide sodium dodecyl sulfate gel. Rabbit antibody 706 against human microvillar membrane, preabsorbed to canine kidney proteins was used. The positions of marker proteins and their molecular masses in kDa are indicated.

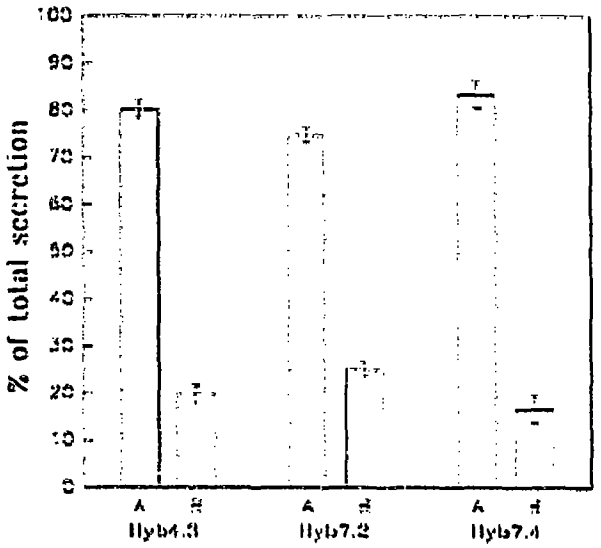

Fig. 3. Secretion of anchor-minus APN and anchor/stalk-minus APN from clonal MDCK-cells. Hyb4.3 (n = 8), Hyb7.2 (n = 6) and Hyb7.4 (n = 11) were grown on filters. The apical (A) and the basolateral (B) media from a 24-h incubation were collected and analyzed for aminopeptidase activity.

is targeted to the apical side as effectively as wild-type APN i.e. 80% on the apical membrane domain [12] (Fig. 3). The Hyb7.2 clone secreted 74.8 ± 1.5% to the apical side and 25.2 ± 1.5% to the basolateral side and the Hyb7.4 clone secreted 83.4 ± 2.8% to the apical side and 16.6 ± 2.8% to the basolateral side. Anchor-minus APN [7] is secreted 80.3 ± 1.8% to the apical side and 19.8 ± 1.8% to the basolateral side (Fig. 3). To verify that the measured activity originated from aminopeptidase N, medium from Hyb7.4 was separately immunoprecipitated with antibodies 705, 706 or an unrelated antibody. Both 705 and 706 were able to immunoprecipitate more than 99% of the aminopeptidase activity in Hyb7.4 medium, whereas the unrelated antibody was unable to immunoprecipitate the aminopeptidase activity.

Previously using a 6 h incubation instead of a 24 h incubation before collection of the media we reported that the anchor-minus APN is secreted 73.0 ± 3.3% to the apical side and 27.0 ± 3.3% to the basolateral side [7]. As shown in Fig. 4 the sorting efficiency of the Hyb4.3 cells appears to improve with time. Similar data were obtained with the clone Hyb7.4 (data not shown). This is not due to transcytosis from the basolateral to the apical membrane since less than 1% of anchor minus APN added to the basolateral side of nontransfected MDCK cells is found on the apical side after 24 h [7]. The apparent improved ability to secrete in a polarized manner during a 24 h period is probably due to recovering of the cell from the handling involved in this type of experiment.

4. DISCUSSION

In the present study we have used the enzymatic activity of the truncated APN to quantitate the recombinant protein. We have previously demonstrated that the aminopeptidase activity measured in this system originates from recombinant human APN [7].

APN has a short cytosolic domain, a transmembrane part and a large ectoplasmic domain. On the basis of negative staining of purified APN inserted into lipid vesicles, it has been shown that the ectodomain of APN consists of a stalk placing the catalytic head group about 5 nm away from the membrane [13]. The three-dimensional structure of APN is not yet known and it is therefore unclear precisely what stretch of amino acids in the primary structure that corresponds to the stalk. We have constructed a secretory form of APN, anchor/stalk-minus APN, by the addition of the hemagglutinin signal peptide including the signal peptidase cleavage site and a linker sequence to amino acids 65–967 of APN. This corresponds to deletion of the cytosolic domain, the transmembrane domain and an approximately 5 nm long α-helix of the aminoterminal part of the ectodomain.

Previously it has been shown that human wild-type APN is transported mainly to the apical membrane when expressed in MDCK-cells [12]. Recently it was shown that anchor-minus APN is secreted to the apical side as efficiently as the wild-type APN and differed from the secretory granule protein, cystatin C [7]. On this basis we concluded that human APN carries an apical sorting signal on its ectodomain [7]. In the pres-

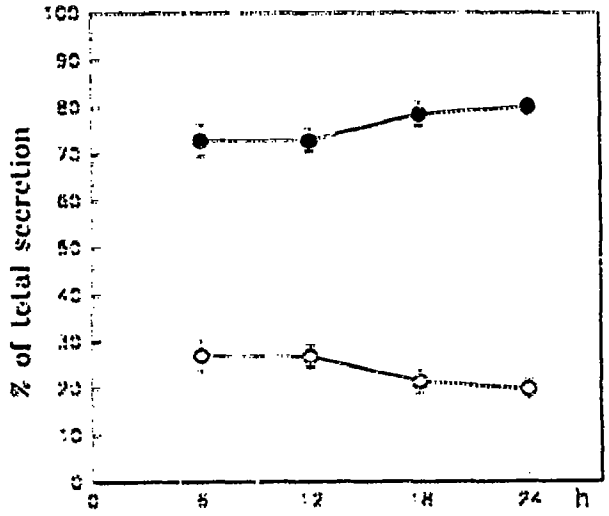

Fig. 4. Time course of sorting efficiency of anchor-minus APN. Hyb4.3 cells were grown on filters. The apical (●) and the basolateral (○) media were collected at different times and analyzed for aminopeptidase activity. n = 5–8.

ent study we have shown that a protein lacking further 31 amino acids of the aminoterminal ectodomain of anchor-minus APN has an unchanged targeting efficiency to the apical side in MDCK-cells. The serine/threonine rich stretch of amino acids 34–64 of human APN is thus not necessary for the correct sorting of APN. We therefore suggest that the apical sorting signal resides between amino acids 65 and 967 of the ectodomain although the formal possibility exists that the linker sequence Ile-Arg-Gly-Ser added to the anchor/stalk minus APN, contains an apical sorting signal.

It is well known that proper folding of proteins is necessary to avoid retention and degradation in the endoplasmic reticulum [22]. Many studies using site-directed mutagenesis or hybrid proteins have been hampered by the fact that the mutated proteins are unable to fold correctly and therefore are retarded and degraded intracellularly. The two mutated forms of APN used in this study has retained enzymatic activity indicating proper folding of the proteins. We were unable to express two hemagglutinin signal peptide–APN hybrid proteins, one consisting of amino acids 1–17 of hemagglutinin, Ile Arg Gly and amino acids 48–967 of human APN and the other consisting of amino acids 1–17 of hemagglutinin, Ile, Arg, Gly and amino acids 90–967 of human APN (unpublished results). This suggests that the requirement for 'proper folding' or 'proper structure' is very delicate, leaving only proper folded complete domains to pass through the endoplasmic reticulum. For this reason we find it likely that the amino acids 34–64 deleted in this study is indeed identical with the stalk domain of the APN molecule and thus that amino acids 65–967 corresponds to the catalytic head group. The distance between the membrane and the head group and the length of the putative stalk domain is compatible with an alpha helix structure of the stalk. In this case the prolines present between amino acids 34–64 of human APN will introduce bends into the helix.

The findings described in this study form the basis for further studies in order to identify the apical sorting signal on the catalytic head group of human aminopeptidase N.

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