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Complete amino acid sequence of human intestinal aminopeptidase N as deduced from cloned cDNA

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The complete primary structure (967 amino acids) of an intestinal human aminopeptidase N (EC 3.4.11.2) was deduced from the sequence of a cDNA clone. Aminopeptidase N is anchored to the microvillar membrane via an uncleaved signal for membrane insertion. A domain constituting amino acid 250–555 positioned within the catalytic domain shows very clear homology to *E. coli* aminopeptidase N and contains Zn^{2+} ligands. Therefore these residues are part of the active site. However, no homology of the anchor/junctional peptide domain is found suggesting that the juxta- and intramembraneous parts of the molecule have been added/preserved during development. It is speculated that this part carries the apical address.

Aminopeptidase N; cDNA cloning; Amino acid sequence; Active site; Sorting signal; (Caco 2 cell)

1. INTRODUCTION

Aminopeptidase N (EC 3.4.11.2) is one of the major proteins of the microvillar membrane of the small intestinal and kidney proximal tubular epithelial cells. The enzyme is also present in the plasma membrane of other cell types like the hepatocytes [1,2].

In epithelial cells of different origin intracellular transport of plasma membrane proteins seems to follow different routes [3,4] indicating that sorting signals and/or receptors are cell type specific. In this context it would be valuable to carry out detailed structural comparisons of analogous plasma membrane proteins like aminopeptidase N expressed in cell types using different pathways for intracellular transport. Molecular cloning of aminopeptidase N from any mammalian tissue is thus the starting point for such studies and also provides the tools for future studies on the regulation of the expression of the aminopeptidase N gene and on the pre-translational and translational events at a molecular level. Structural studies might as well reveal the localisation of the active site in the polypeptide chain.

This is the first report on the isolation and characterization of a mammalian aminopeptidase N cDNA clone. The clone covers the complete coding region and the primary structure of the protein has been deduced from the cDNA sequence.

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2. EXPERIMENTAL

2.1. *Sequence analysis of the amphiphilic and the 61 kDa fragment of pig aminopeptidase N*

The amphiphilic form of pig aminopeptidase N was purified

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from the small intestine of pigs having had their pancreas disconnected 3 days prior to slaughter [5]. Removal of detergent and concentration were accomplished on a DEAE-cellulose column before amino acid microsequencing was carried out.

The proteolytic C-terminal 61 kDa fragment of pig aminopeptidase N was purified by an immunological technique from a preparation of aminopeptidase N obtained from normal pigs in which the proteolytically clipped aminopeptidase N is present [5]. About 2 mg of pure aminopeptidase N in 0.05% LiDS was boiled for 5 min to dissociate the polypeptide chains and Triton X-100 was then added (0.1% final concentration). The N-terminal 123 kDa fragment and the intact enzyme was then removed on an immunoadsorbent Sepharose column, derivatized with an antibody specifically directed against the N-terminal 123 kDa fragment [6] and equilibrated with 50 mM Tris-HCl, pH 8.0, containing 0.3 M LiCl and 0.1% Triton X-100. The C-terminal 61 kDa fragment was collected in the break through volume and analyzed for purity by SDS-PAGE [7]. Traces of rabbit IgG were removed by passage through a Protein A Sepharose column (Pharmacia, Uppsala, Sweden) and the detergents were removed by Extractigel (Pierce, Rockford, IL). The sample was concentrated by Ultrafiltration (Amicon Corp., Danvers, MA) and dialysed against 50 mM $(NH_4)_2CO_3$.

About 1 nmol of the amphiphilic enzyme and of the C-terminal 61 kDa fragment were subjected to automatic amino acid sequence analysis on a gas-phase sequencer (Applied Biosystems, model 470A) using the program '02NRUN' [8]. The PTH-derivatives were analysed on a Waters HPLC system using a Spherisorb ODS II column developed by a methanol-ethanol gradient so all derivatives were separated.

2.2. *Intestinal mucosa cDNA libraries*

Initially a cDNA library in λgt11, established using poly(A^+) mRNA obtained from rabbit small intestine was used [9,10]. In the process of searching for a full-length clone a human intestinal cDNA library was created according to the same principles [9] using poly(A^+) mRNA purified from Caco 2 cells grown to confluence.

2.3. *Screening of the cDNA libraries*

Immunological screening of the rabbit library was carried out essentially as described by [9]. The antibody used was raised in guinea pig using a purified rabbit aminopeptidase N as antigen. This rabbit aminopeptidase N was after papain release from the intestinal microvillar membranes [11] purified by gel filtration (Ultrogel 22) and its purity analyzed by SDS-PAGE [7]. The antibody was fairly specific as analyzed by rocket immunoelectrophoresis using histochemical aminopeptidase N staining followed by protein staining [12]. The filters were blocked by 2% Tween 20 (5 min) prior to incubation with the primary antibody [13]. The second antibody was a peroxidase-conjugated rabbit anti-guinea pig IgG (Dako A/S, Copenhagen, Denmark). The filters were finally washed in Na-acetate, pH 5.2, and stained with 3-amino-9-ethylcarbazole. Positive plaques were picked and purified.

The immunological screening of about 100000 recombinants of the rabbit λgt11 library resulted in isolation of 8 clones. One clone gave a stronger immunological signal than the others and was therefore purified and the DNA isolated. In Northern blotting (fig.1) it hybridized to one single 3.2 kB mRNA which is a size sufficient to code for the aminopeptidase N polypeptide chain (115 kDa; [14]). To substantiate further the identity of the clone the β-galactosidase fusion protein was expressed in Y1089 *E. coli* cells and analyzed by Western blotting. It showed strong reactivity with the antiserum directed to the rabbit as well as to the pig (C-terminal fragment [6]) aminopeptidase N (not shown).

Screening using the radiolabelled rabbit 5′-*Eco*RI fragment by in situ plaque hybridization was carried out according to the principles delineated by Maniatis et al. [15].

2.4. *Subcloning of cDNA inserts in plasmids*

Lambda DNA was purified, after having isolated the phages from lysates produced in a liquid NZCYM-medium [15]. The cDNA insert was excised by digestion with *Eco*RI, isolated by agarose gel electrophoresis and ligated into the appropriate Bluescript plasmid (Stratagene, San Diego, CA). The constructs were used to transform [16] competent XL-Blue-1 cells (Stratagene, San Diego, CA). Selection took place on X-gal/IPTG/ampicillin/tetracyclin-LB plates.

Recombinants were isolated and the plasmid DNAs were analyzed on agarose gel electrophoresis. CsCl-banded plasmid DNA containing the inserts of interest was prepared.

2.5. *DNA sequencing*

The cloned *Eco*RI fragments (in the appropriate Bluescript$^+$ plasmid and direction) were deleted either by using internal restriction enzyme sites or by creating nested deletions by the *Exo*III/Mung bean method [17]. Single-stranded DNA was, after transformation of XL-Blue-1 cells, prepared from cultures after addition of a suitable helper phage (R408, Stratagene, San Diego, CA).

DNA sequencing was carried out using the dideoxy sequencing method [18] using either the Klenow fragment or the T_7 DNA polymerase (Sequenase, USB, Cleveland, OH). [α-^{35}S]dCTP or [α-^{35}S]dATP was used as radiolabel and the analysis was carried out using salt gradient polyacrylamide gels [19].

For the purpose of initial characterisation of clones sequencing was in some instances carried out on double-stranded, supercoiled plasmid DNA [20].

The full-length human aminopeptidase N clone (and the partial rabbit clone) was sequenced (in both directions when appropriate). For all stretches 3–9 sequence reactions were carried out and analyzed.

The PC-GENE software (Genofit, Grand-Lancy, Switzerland) was used to organize and analyze the DNA sequences and to predict the membrane spanning segment. The homology studies were carried out using the ALIGN and DOT-MATRIX programs of the PIR software (NBRF, Georgetown University Medical Center, Washington, DC).

3. RESULTS AND DISCUSSION

3.1. *Isolation of a clone covering the complete coding region of human aminopeptidase N*

The search for a complete aminopeptidase N

clone was carried out using a radiolabelled 1.2 kbp *Eco*RI fragment of the rabbit clone.

Both the rabbit library (approx. 150000 recombinants) and the human library (approx. 120000 recombinants) were screened.

The insert of one of the human clones isolated hybridized to a 3.2 kb mRNA (fig.1) and contained at one end a DNA sequence coding for an amino acid sequence similar to the N-terminus determined for the intact amphiphilic pig aminopeptidase N (this paper, fig.3) and also similar to that published for the rabbit enzyme [21]. The designated ATG start codon (position 25) is the first one found in the nucleotide sequence (fig.2) and it is surrounded by a nucleotide structure making it effective for initiation by eukaryotic ribosomes [22]. It is positioned at the beginning of a 2901 nucleotides long open reading frame ending at the TAG stop codon starting at position 2926.

By complete sequencing the human clone was shown to consist of 3370 bp (fig.2). A stretch starting at nucleotide 1759 codes for an amino acid sequence found in the N-terminal end of the 61 kDa C-terminal fragment (fig.3) of the pig aminopeptidase N further proving the authenticity of this clone. At position 3362 there is a typical polyadenylation signal (AATAA) although no poly(A) containing stretch was found in the 3′-end. The molecular mass of the deduced protein sequence is 110 kDa, a value close to that of the in vitro translation product of the pig [14] and rabbit (not shown) aminopeptidase N. Thus this clone covers the complete coding region of aminopeptidase N.

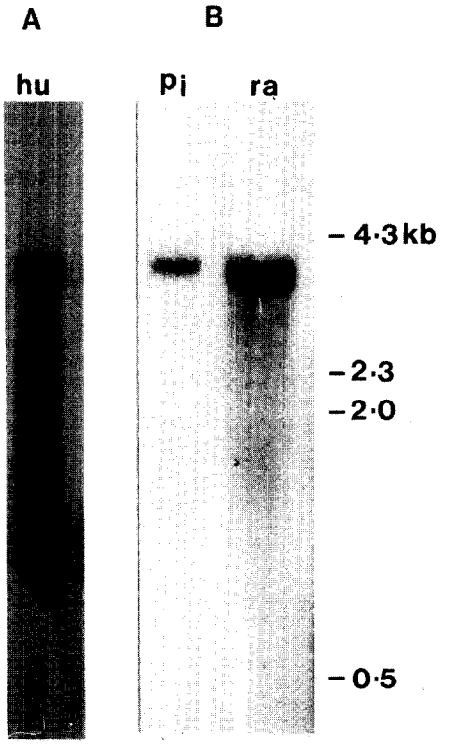

Fig.1. Size determination of aminopeptidase N mRNA by agarose electrophoresis followed by Northern blotting. RNA was isolated from human (hu; type of donor: see [59]), pig (pi) and rabbit (ra) jejunum. After denaturation in formamide the RNAs were electrophoresed in formaldehyde containing agarose gel (1%), transferred to nitrocellulose membranes and probed with the nick-translated 5′-*Apa*I fragment of the human clone (A) or a 1.2 kb fragment of the rabbit clone (B).

3.2. *Structure of the catalytic domain*

The polypeptide chain of the catalytic domain (amino acid residue 70-967; see later for explanation) contains 10 possible Asn-X-Ser/Thr *N*-glycosylation sites (fig.3). In vitro translation experiments in the presence of microsomal membranes [23] have shown that most of these sites are glycosylated. Aminopeptidase N is tyrosine sulfated [24]. Accordingly two potential sulfation sites are found (Tyr^{176} and Tyr^{913}) fulfilling 4 out of 5 criteria for this type of sulfation [25].

Analysis for internal homology by the PIR DOTMATRIX program comparing intestinal aminopeptidase N to itself indicated vaguely that gene duplication might have occurred. This is further strengthened by a similar finding for the corresponding *E. coli* enzyme. PIR ALIGN comparison of human aminopeptidase N residues 69–480 to residues 481–900 gave a score of 3.1 SD (matrix bias +2, gap penalty −8). Intestinal aminopeptidase N thus in this respect bears some similarities to sucrase-isomaltase, another intestinal microvillar enzyme [10].

Comparison of human intestinal aminopeptidase N to the *E. coli* aminopeptidase N [26,27], a cytoplasmic enzyme involved in intracellular protein turnover, revealed a significant homology (figs. 3 and 4). Especially the stretch 250–555 in intestinal aminopeptidase N displayed pronounced similarity to the stretch between residue 156 and 451 in the *E. coli* enzyme. Statistical analysis by the

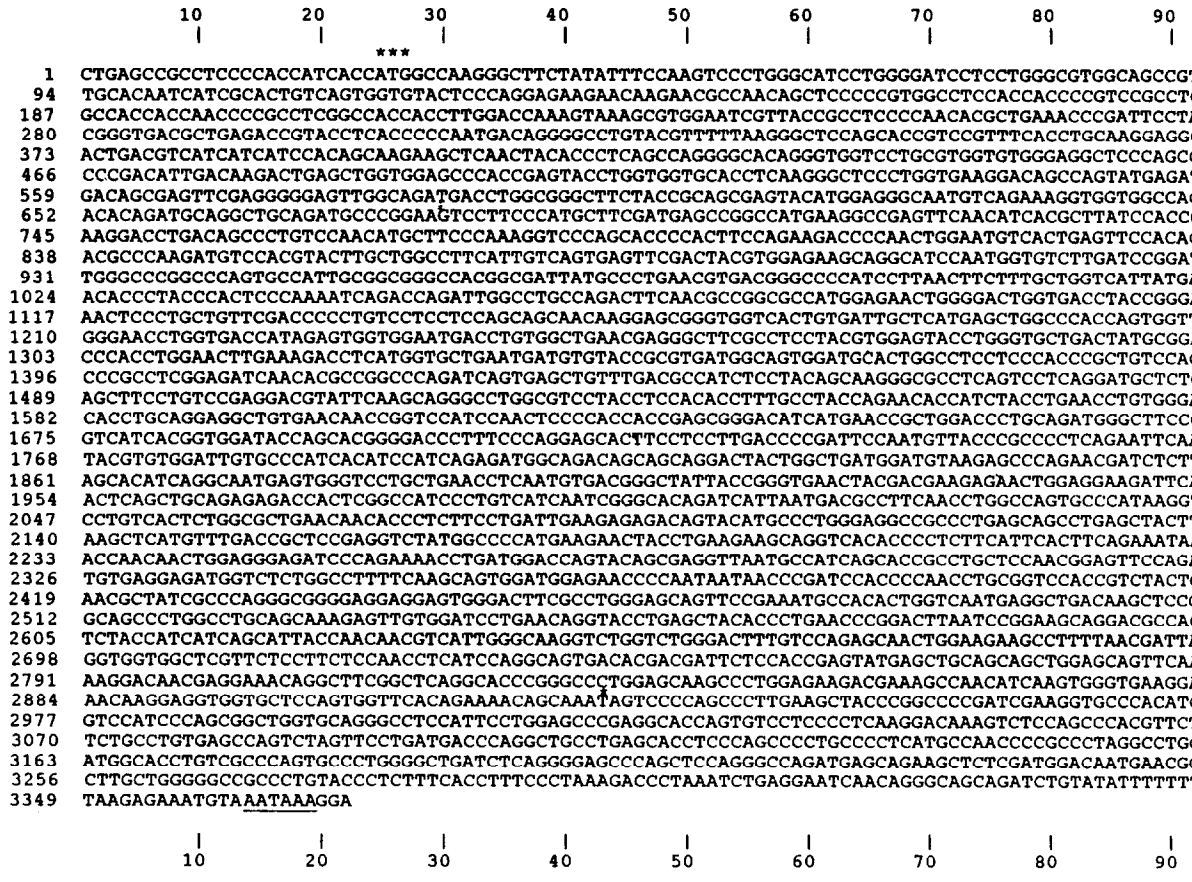

Fig.2. DNA sequence of human intestinal aminopeptidase N. The initiator ATG is marked ***, the stop codon TAG indicated with * and the polyadenylation signal is underlined.

PIR ALIGN program gave a value of 15.0 SD confirming the common evolutionary origin. This highly conserved segment, predicted to constitute the functional peptidase domain, is rich in potential Zn^{2+} ligands such as histidine and glutamic acid (fig.3). This finding fits well with the fact that aminopeptidase N is a Zn^{2+} containing enzyme [28,29]. The conserved sequence HEXXH, also found in thermolysin [30], endopeptidase 24.11 [31,32] and collagenase [33], was easily recognised and is thus with high probability part of the active site.

3.3. *Structure of the anchoring domain*

Using the conformational preference parameter of Rao and Argos [34] for predicting intramembranous helices of proteins only Lys^{9}–Val^{32} of the aminopeptidase N sequence fulfilled the criteria. The sequence contains a 23 amino acid long hydrophobic stretch starting at Leu^{10} and ending with Val^{32}. This forms the membrane spanning part. Charge shift electrophoresis and hydrophobic labelling have shown the existence of a hydrophobic domain in the molecule [5,35]. It has earlier been demonstrated to be positioned in the N-terminal part of the molecule [36,37] anchoring the catalytic headgroup to the membrane. Due to its total length it can cross the membrane only once. The catalytic domain is positioned extracellularly as it can be released from the membrane by proteolytic treatment leaving the hydrophobic N-terminal part in the membrane. Consequently amino acids 2–9, starting with the N-terminal alanine (the initiator methionine is likely to be cleaved off posttranslationally as this residue is absent at the pig and the rabbit N-

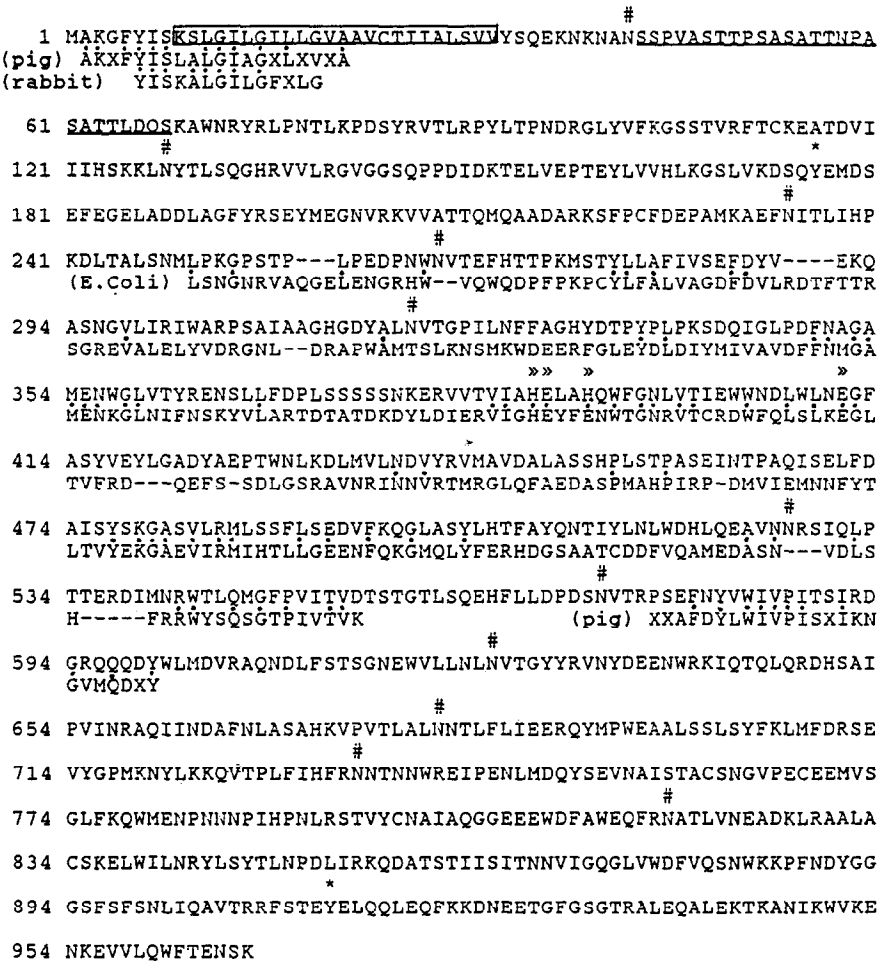

Fig.3. Alignment of the amino acid sequences of human intestinal aminopeptidase N and *E. coli* aminopeptidase N. The amino acid sequence deduced from the cDNA sequence (fig.2) is shown aligned with part of the *E. coli* aminopeptidase N (amino acids 252–451) [26,27] predicted to constitute the functional peptidase domain. In addition the partial amino acid sequences of the rabbit [21] and pig enzyme (this paper) are shown. Boxed amino acids show the membrane spanning part; underlined amino acids show the Ser/Thr-rich domain of the junctional peptide; #, potential glycosylation sites; *, potential sulfation sites; », predicted ligands of Zn^{2+}.

termini), are positioned on the cytoplasmic side of the membrane, all in accordance with results from cytosolic labelling of aminopeptidase N [38,39]. The cytosolic and transmembrane domains have an overall structure compatible with that of other N-terminally anchored endogenous microvillar proteins [10,31,32,40,41] in which the cytosolic and transmembrane parts also function as the molecular signal for endoplasmic reticulum segregation without being cleaved. This phenomenon is also seen for a few other plasma membrane proteins (review [42]). It is interesting to notice that all the cytoplasmic parts of the N-terminally anchored microvillar proteins so far studied have the same net charge (+3). This might be of importance for the correct insertion and final topology for these proteins as change in charges in the corresponding part of cytochrome P-450 have been shown to heavily influence its orientation in the membrane [43].

The earlier described similarity between human and *E. coli* aminopeptidase N does not encompass the first 70 residues (fig.4) which thus might be considered as an addition/preservation during

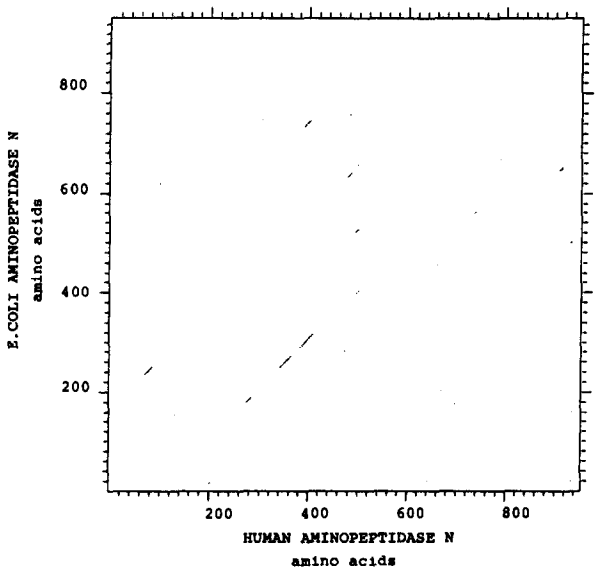

Fig.4. Comparison of human and *E. coli* aminopeptidase N. Using the PIR DOTMATRIX program (minimum score 30; window size 30) the complete amino acid sequences of human microvillar aminopeptidase N (this paper) and *E. coli* aminopeptidase N [26,27] were compared.

evolution as the intestinal aminopeptidase N is 97 amino acids longer than the *E. coli* enzyme [26,27]. This extra part of the molecule includes the 31 residue long cytoplasmic and transmembrane part and thus also an approx. 40 amino acid long stretch, representing the 5 nm long junctional peptide bridging the gap between the membrane and the catalytic domain [44]. The junctional peptide has a very high content of serine and threonine. The stretch Ser^{43}–Ser^{68} has a Ser/Thr content of 50% and it also contains one potential Asn glycosylation site. A similar domain has also been detected in exactly the same position in relation to the membrane on the pro-sucrase-isomaltase [10]. The LDL-receptor [45] and glycophorin [46], although both having their C-terminus on the cytoplasmic side, have similar, although shorter, externally positioned juxtamembranous Ser/Thr-rich domains. The importance of these Ser/Thr-rich domains are not extensively elucidated but the domain has been shown to be without influence for the proper function of the LDL-receptor [47].

Interestingly, one N-terminally anchored protein: intestinal aminopeptidase N (this paper) and one C-terminally anchored protein: intestinal [48] and placental [49] alkaline phosphatase show significant homologies to their corresponding *E. coli* enzymes. However the homologies do not encompass the juxta- and intra-membraneous parts of the enzymes (fig.4). These additional segments (C- or N-terminally localized) anchoring the proteins to the membrane might also carry the signal for the apical transport and localization. It seems clear that the N-terminal anchoring is not a prerequisite for the microvillar expression as C-terminally anchored proteins like alkaline phosphatase [48] and lactase-phlorizin hydrolase [50] can as well be effectively expressed in the microvillus membrane. The localisation of the apical signal might then be ascribed to structures in the catalytic domain of the microvillar enzymes. However, this is a less attractive hypothesis as a lysosomal α-glucosidase shows general strong homology to the microvillar enzyme sucrase-isomaltase [51] and in addition shows no similarity to the stretch corresponding to the intramembraneous and juxtamembraneous parts of sucrase-isomaltase. Some microvillar enzymes are bound to the membrane via phosphatidylinositol [52,53] and therefore probably have a C-terminal hydrophobic amino acid sequence split off in the endoplasmic reticulum during biosynthesis [54]. If present outside the catalytic domain the microvillar signal therefore should be positioned in the junctional peptide connecting the catalytic domain to the transmembrane and cytosolic parts of the anchor. We therefore looked specifically for sequence similarities to amino acid residues 30–59 (i.e. the junctional peptide segment) in the Swiss Protein Data base (release 06) using the program SCANSIM. A similarity (8 out of 15 amino acids identical) to the amino acid residues 221–235 in human IgA α_1 heavy chain [55] was found. Using the PC-GENE PCOMPARE the similarity was found significant (4.7 SD units). This segment corresponds to the hinge region in the heavy chain of IgA and is situated between regions $C\alpha_1$ and $C\alpha_2$. The region has been proposed to take part in the interaction with the pIg receptor [56] mediating the transport of IgA from the basolateral to the apical membrane. This route has been demonstrated to be used for hepatocyte aminopeptidase N [57]. The finding therefore fits into our earlier proposal [58] that this receptor might be involved in the transport of membrane proteins from the basolateral to the microvillar membrane.

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