Biosynthesis and polarized distribution of neutral endopeptidase in primary cultures of kidney proximal tubule cells

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When cultured in defined medium, kidney proximal convoluted tubule (PCT) cells form a homogeneous population and retain a number of differentiated functions. To characterize this cell system further as a functional model of epithelial polarity, we investigated the biogenic pathway of neutral endopeptidase (NEP), one of the most abundant microvillar membrane proteins in intestinal and kidney cells. We showed that, in contrast with some tumoral cell lines, RNA extracted from PCT cells shows the presence of a single mRNA species encoding NEP. Pulse-chase studies followed by selective immunoprecipitation of NEP mole-

cules present either at the cell surface or in intracellular cell compartments showed that newly synthesized NEP molecules reached the cell surface as early as 30 min after the beginning of the chase with maximum cell surface expression at 60 min. When grown on semipermeable supports, PCT cells were found to target NEP exclusively to the apical plasma membrane. Similar results have been described using MDCK cells to study targeting of recombinant NEP. Thus primary cultures of PCT cells represent a new model with which to investigate the biogenic pathway of endogenous proteins in native epithelial cells.

INTRODUCTION

Epithelial cells perform a variety of specialized functions that are reflected by the polarized morphology and organization of their surface membrane domains, the so-called basolateral and microvillar (apical or brush-border) membranes. These domains possess unique sets of integral membrane proteins.

The important physiological role of epithelial cells has stimulated interest in biosynthetic events underlying the microvillar expression of hydrolytic enzymes that serve as protein models for the study of cell polarity. Apart from a few reports on liver plasma-membrane proteins [1], information on the biosynthesis of endogenous plasma-membrane proteins has generally come from polarized epithelial cells of the intestine and kidney. Established epithelial cell lines such as Caco-2 [2] and Madin-Darby canine kidney (MDCK) cells [3] have been used extensively to investigate microvillar enzyme biosynthesis in the intestine and kidney respectively [4,5]. The results indicated that some epithelial cell types sort apical and basolateral proteins in a second sorting site, the basolateral membrane or the basolateral endosome, and that protein targeting is highly dependent on both cell and protein type.

To determine whether the results observed with infected MDCK cells are also observed with native epithelial cells, we investigated the biogenesis of endogenous protein in primary cells of renal origin. Primary cells of proximal convoluted tubules (PCT) represent a functional model of epithelial polarity. Although PCT cell cultures are widely employed for a better understanding of the physiological and pathological properties of cells *in situ*, their exact biochemical features have not yet been fully elaborated. Previous work in our laboratory has established that culturing PCT cells in defined media provides a homogeneous population with attachment, proliferation and growth as well as long-term expression of polarity [6]. The maintenance of high specific activities of maltase, trehalase, γ -glutamyltransferase,

alkaline phosphatase and neutral endopeptidase (NEP, neprilysin, endopeptidase 24.11) in primary cultures suggests that cultured cells actively synthesize these enzymes. Therefore such cells constitute an excellent system for further characterization of the processing and translocation steps associated with the biosynthesis of glycoproteins. In this context, NEP as a major microvillar membrane peptidase in PCT (for review see Kenny [7]) is an ideal candidate to deliver an insight into the mechanisms and events that are responsible for the expression of microvillar enzymes in the apical portion of plasma membranes. We have previously reported on the biosynthesis and targeting of this enzyme in MDCK cells [8]. In this cell line recombinant rabbit NEP is synthesized as a high-mannose form (88 kDa) which is processed to a mature complex glycosylated 94 kDa protein directly targeted to the apical cell surface.

In the present study, we investigated the biogenic pathway of NEP in kidney PCT cells cultured in Petri dishes and semipermeable collagen-treated filters. Culturing cells on filters allows accessibility to their basal and apical surfaces for biochemical analysis and experimental manipulation. We showed that PCT cells growing on permeable supports target NEP exclusively to the apical plasma membrane and that the biogenic pathway of NEP in these cells is similar to that described *in vivo* [9] and in MDCK cells [8].

MATERIALS AND METHODS

Cell culture media

A 50/50 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium was used throughout these studies. This basal culture medium was supplemented with 15 mM Hepes buffer (pH 7.4), 1.2 mg/ml NaHCO_3 , 50 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 5 mM glutamine and 1% non-essential amino acids. The other growth supplements (Collaborative Research) added to the medium just before use include bovine

Abbreviations used: PCT, proximal convoluted tubule; NEP, neutral endopeptidase (EC 3.4.24.11); MDCK cells, Madin-Darby canine kidney cells; $\times$ SSC, 0.15 M NaCl/0.015 M sodium citrate.

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insulin (5 µg/ml), human transferrin (5 µg/ml), hydrocortisone (50 mM) and selenious acid (5 ng/ml).

Initiation of primary cultures of rabbit kidney PCT

Kidneys were removed aseptically from specific pathogen-free New Zealand White rabbits (1.5–2 kg) and immediately transferred to ice-cold basal culture medium. They were then decapsulated and cut in half; the cortex was separated from the medulla and sliced, using a Stadie–Riggs microtome. The cortical slices of two kidneys were pooled in ice-cold basal culture medium, washed three times with the same medium, and resuspended in a 250 ml Erlenmeyer flask containing 30 ml of this medium and 0.15 g of collagenase/100 ml (grade II; *Clostridium histolyticum*; Boehringer). After 2 min of passage in 95% O₂/5% CO₂, the flask was agitated for 30 min at 37 °C in a shaking water bath. The collagenase-digestion step was terminated by the addition of cold culture medium, and the tissue mixture was filtered through a 150 µm-pore-size stainless-steel filter (Canadian Standard Sieve Series). The suspension of renal tubules was then gently centrifuged for 30 s at 60 g and this procedure was repeated three more times in cold basal culture medium. The final pellet was weighed and 1.2 g of tissue was resuspended in 30 ml of 50% Percoll solution to achieve a separation of tubule fragments by the method of Vinay et al. [10]. The F4 layer, made at 98% of proximal tubules, was then removed from the Percoll gradient and washed three times with cold basal culture medium at the same low centrifugation speed as above. The proximal tubules were finally resuspended in growth culture media and seeded at a density of 0.42 mg/cm² in 35 × 10 mm Falcon Petri dishes (Becton Dickinson Labware) or on permeable supports (collagen-treated Costar membranes from Transwell) in Costar's Transwell cell culture chamber inserts (Cambridge). A portion of the tubule suspension was frozen to serve as an internal standard for normalization of the enzyme data. Cell culture was performed in a humidified 5% CO₂/95% air at 37 °C. The growth medium was first changed after 2 days, a time period found to be sufficient for cell colonization in the plastic Petri dishes and filters. It was then changed on alternate days.

Pulse-chase studies

Pulse-chase studies were performed 7–9 days after seeding. Cells grown on Petri dishes were washed with RPMI medium lacking methionine (Gibco Laboratories) and maintained in this medium for 1 h before the pulse. [³⁵S]Methionine (100–150 µCi/ml) (Amersham Corp., specific radioactivity 1192 Ci/mmol) was then added to the medium for 20 min. After the pulse, monolayers were incubated in 1 ml of RPMI medium containing methionine at a concentration of 0.15 mg/ml (Sigma) for increasing time periods.

Cell surface immunoprecipitation

After pulse-chase labelling, the cells grown in Petri dishes were washed twice with ice-cold PBS containing BSA (1%) and incubated for 1 h at 4 °C in the same buffer with a 1:200 dilution of polyclonal antibody added to the apical surface. This allowed the selective immunoprecipitation of newly synthesized NEP molecules that had reached the apical domain of the plasma membrane. After the incubation period, monolayers were washed twice with 1 ml of cold PBS. The cells were then scraped from the

dishes and lysed in 100 µl of PBS buffer containing 1% octyl glucoside (1-*O*-*n*-octyl β-D-glucopyranoside). Nuclei were removed by centrifugation at 1400 g for 20 min.

The postnuclear supernatants were diluted in 0.5 ml of immunoprecipitation buffer (10 mM Tris/HCl, pH 7.4, 50 mM NaCl, 1 mM methionine, 1 mM phenylmethane sulphonyl fluoride, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 0.1% BSA, 100 kallikrein inhibitory units/ml aprotinin) and transferred to a tube containing Protein A–Sepharese beads (Pharmacia). To recover the immunoprecipitated antigens, the beads were boiled in sample buffer for 5 min, centrifuged for 1 min at 1400 g and analysed by SDS/PAGE.

Surface localization of NEP in PCT cells

PCT cells were grown to confluence in 24 mm Transwell chambers, chilled to 4 °C, rinsed with cold PBS and incubated in PBS containing 0.5% BSA for 1 h with radiolabelled monoclonal antibody 2B12 (5 × 10⁶ c.p.m./ml) [11]. Antibody solutions were added to either the apical or basolateral compartment or both of the filter unit. The opposite compartment was filled with PBS/BSA containing 10 µl/ml immunobeads coupled to rabbit anti-mouse immunoglobulins to prevent artifacts caused by possible disruption of the monolayers. After incubation, both sides of the monolayers were washed three times with cold PBS. Cells scraped from the filters were solubilized in 1% octyl glucoside and electrophoresed in 8% acrylamide as described by Laemmli [12]. Autoradiograms of the slabs were scanned with an enhanced laser densitometer (LKB Ultrascan XL), using the GELSCAN XL program, and binding of the antibody was quantified.

Northern-blot analysis

Total RNAs were prepared from total rabbit kidney, PCT, PCT cells, skeletal muscle and MDCK cells expressing recombinant rabbit NEP [8] (MDCK NEP) by the method of Chomczynski and Sacchi [13]. RNA samples were fractionated on 1% denaturing agarose gel, transferred to a nylon membrane (Hybond, Amersham, Oakville, Canada) and probed with ³²P-*Bst*EII/*Xba*I-pSVENK19 cDNA fragment (specific radioactivity 8 × 10⁴ c.p.m./ng). Vector pSVENK19 was constructed by inserting an *Hpa*I fragment containing all NEP-coding sequences downstream of the simian virus 40 early promoter in plasmid pSVP3 [14] as shown schematically in Figure 2. The probe used encompasses the sequence coding for part of the ectodomain including the Zn-binding site of the enzyme. The probe was synthesized by the method of Feinberg and Vogelstein [15]. Briefly, the DNA to be labelled was first denatured and then mixed with oligodeoxyribonucleotides of random sequence. These random oligomers anneal to random sites on the DNA and serve as primers for DNA synthesis by the Klenow enzyme (Pharmacia). The blots were washed in 0.2 × SSC (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) buffer at 25 °C for 15 min and with 0.1 × SSC at 55 °C for 30–45 min, then prepared for autoradiography. To monitor loading, the same membrane was probed with a ³²P-labelled actin cDNA isolated from neurons of *Aplysia californica* [16].

Immunocytochemistry

For immunogold labelling of NEP present at the cell surface, PCT cells cultured in plastic Petri dishes were fixed for 45 min at 4 °C in 100 mM cacodylate buffer, pH 7.4, containing 2% (v/v) glutaraldehyde (JBS-Chem), and washed by three 10 min incubations

tions in the same buffer. They were scraped from their supports, incubated for 30 min in PBS/BSA at room temperature and then for 45 min in the same buffer with specific polyclonal antibody raised against rabbit NEP. After washing, a colloidal gold-conjugated anti-guinea-pig immunoglobulin fraction diluted in PBS/BSA was added to the cells and the samples were left for 1 h at room temperature. After three washes in PBS, the cells were centrifuged and resuspended in cacodylate buffer containing 4.5% filtered sucrose and postfixed for 1 h on ice with 1% OsO₄ in PBS. They were then washed with PBS and dehydrated with increasing ethanol concentrations. The fixed cells were embedded with Epon B resin and, after polymerization at 60 °C, resin cylinders were cut with the ultramicrotome.

RESULTS

Morphological and functional polarity of PCT cells

Transmission electron microscopy was used to study the ultrastructural features of epithelial cells cultured on Petri dishes. As shown in Figure 1, these included distinct apical and basal cell surface domains that respectively marked the luminal and basal stroma (basal lamina). In addition, extensive membrane interdigitations and junctional complexes were characteristic of lateral aspects of the cell surface. Colloidal gold labelling showed that NEP were mainly localized on microvilli of the apical surface of cells (Figure 1a). The basolateral surface was very weakly labelled around the interdigitations (Figure 1c).

RNA analysis of NEP in PCT and PCT cells in culture

Recent studies have documented more than one NEP mRNA species in the kidney and brain [17] as well as in some tumoral cell lines [18,19]. We took advantage of our cell system to analyse NEP mRNA in a homogeneous cell population of the kidney and to determine whether NEP transcripts from PCT resemble those in the total kidney. The total RNA samples were probed by Northern-blot analysis with a 1700 bp cDNA fragment obtained by digestion of pSVENK19 vector with *Bst*EII and *Xba*I and labelled as described in the Materials and methods section. In total rabbit kidney, PCT and PCT cells, the 3.4 kb transcript was the major NEP mRNA observed (Figure 2, lanes 1, 3 and 4). The low amount of NEP mRNA in PCT cells (lane 4) could be explained by the partial loss of the differentiated functions of these cells after 7 days in culture as previously reported [6]. MDCK cells expressing recombinant NEP were used for comparison purposes (lane 5). To rule out the possibility that variations in the NEP mRNA levels seen on the blot (Figure 2c) could be due to mRNA losses during sample preparation, the same blot was hybridized with an actin probe from *Aplysia californica*. As seen in Figure 2(b) the intensity of the actin band was approximately equal in all samples except in muscle where it was much more intense and found at different positions. This was in agreement with the strong expression of actin isoforms in this tissue. Overall these results suggest that variations in NEP mRNA levels observed in different cell extracts reflect genuine differences in NEP gene expression and not experimental variations during sample preparation and handling.

Polarized distribution of NEP in PCT cells

Primary cultures of PCT cells can grow on permeable supports to form a monolayer that resembles that of the established polarized epithelial cell line MDCK. To quantify more accurately the steady-state distribution of NEP at the cell surface of primary

cells, we performed binding experiments with radiolabelled antibodies added to either the apical or basolateral domain or both of the plasma membrane. It has been shown that antibodies have access to the basolateral membrane of cells grown on Costar filters. We have also shown previously using the same filter units that anti-gp23 antibody freely binds to the antigens present at the basolateral and apical surfaces in MDCK cells [8]. To analyse the distribution of NEP between the basolateral and the apical membrane of cultured PCT cells, filter-grown monolayers were incubated with ¹²⁵I-2B12 antibody raised against rabbit NEP. After extensive washing to remove excess antibody,

Figure 1 Electron microscopy of PCT grown on a plastic support

For immunogold labelling and electron microscopy, cells were processed as described in the Materials and methods section. The bar represents 2 µm; (b) represents a typical PCT cell from a confluent monolayer; (a) and (c) are enlargements of the upper and lower boxed areas in (b). Gold particles are found mainly localized on the microvilli of the apical plasma membrane (a), whereas little label is bound to the basolateral surface (c).

Figure 2 Northern-blot analysis of NEP mRNA in PCT cells

Vector pSVENK19, containing the insert encoding NEP, was constructed by inserting an *HpaI* fragment containing NEP-coding sequences downstream of the simian virus (SV)40 early promoter [14]. The fragment used for probe synthesis was excised with restriction enzymes *BstEII* and *XbaI*. The relevant restriction sites in the vector are as shown in (a). Equal amounts of total RNA (5 µg) extracted from total rabbit kidney (lane 1), rabbit skeletal muscle (lane 2), PCT (lane 3), PCT cells (lane 4) and MDCK NEP cells (lane 5) were electrophoresed on a formaldehyde-agarose gel, blotted on to a Hybond membrane. The membrane was hybridized with either an actin probe cloned from *Aplysia californica* [16] to monitor the amount of mRNA loaded into each track (b) or a ³²P-labelled rabbit NEP cDNA probe (c). Lane 1 shows that rabbit kidneys express one major NEP mRNA species, which was also found in lower amounts in purified proximal tubules (lane 3). Much smaller quantities of the same mRNA were found in cultured cells from proximal tubules (lane 4). MDCK NEP cells stably expressing the recombinant rabbit NEP [8] were used for comparison purposes (lane 5). They also contained low amounts of NEP mRNA with a size similar to that found in lanes 1, 3 and 4. No signal was detected in skeletal-muscle sample (lane 2). The size markers used are ribosomal RNA 28S and 18S.

portions of cell lysates were analysed by SDS/PAGE. The binding of antibodies to NEP molecules was then estimated by SDS/PAGE and fluorography of dried gels. As shown in Figure

Figure 3 Surface localization of NEP in PCT cells

PCT cells grown to confluence in 24 mm Transwell chambers were chilled to 4 °C and incubated in PBS/BSA for 1 h with appropriate dilution of radiolabelled monoclonal antibody (5×10^6 c.p.m./ml). The antibody solution was added to either the apical (lane 1) or basolateral (lane 2) compartment or both (lane 3). Labelled cells were solubilized and electrophoresed in 8% acrylamide. Radioactive light and heavy chains of immunoglobulins are indicated.

Figure 4 Pulse-chase analysis of NEP synthesis

PCT cells grown on Petri dishes were labelled for 20 min with [³⁵S]methionine as described in the Materials and methods section, and incubated with chase medium containing unlabelled methionine for the indicated time. NEP was immunoprecipitated from the solubilized cells and analysed by SDS/PAGE. A fluorogram of the gel is shown. The position of standard rabbit NEP containing complex-type carbohydrate side chains has been indicated. The positions of standard markers are also shown.

3, over 95% of the radioactivity associated with NEP was concentrated at the apical domain (lane 1). Only 5% of radio-labelled antibodies were associated with the basolateral membrane. When antibodies were added to both cell surfaces, the amount of radiolabelled antibodies bound to antigens was not significantly different from that observed at the apical surface (Figure 3, lane 3). Taken together these results show the exclusive presence of NEP at the apical membrane of culture PCT cells.

Biosynthesis and maturation of NEP in PCT cells

It has been demonstrated that our culture conditions provide a homogeneous PCT cell population that expresses 65% of the NEP activity present in the parent tubules [6]. We used this cell system to investigate the biosynthesis and transport of NEP. PCT cells growing in Petri dishes were pulse-labelled with [³⁵S]methionine and chased for increasing times in the presence of excess unlabelled methionine. Newly synthesized NEP molecules were recovered by immunoprecipitation and separated by SDS/PAGE. Labelled NEP molecules recovered after the pulse had a molecular mass of 88 kDa (Figure 4, lane 1). After 15 min chase (lane 2), the molecular mass of NEP increased to 94 kDa, and this increase was correlated with a loss of sensitivity to endoglucosaminidase H (results not shown), suggesting processing from high-mannose to a complex oligosaccharide. At 60 min,

Figure 5 Cell surface appearance of newly synthesized NEP

After pulse-chase labelling, monolayers of PCT cells grown on Petri dishes were incubated for 1 h at 4 °C with polyclonal antibody added to the apical surface. After the incubation period, cells were rinsed and lysed. Immune complexes containing NEP present at the cell surface were directly recovered by adding Protein A-Sepharose beads to the supernatants. The position of standard rabbit NEP has been indicated.

chase, only the mature form of newly synthesized NEP molecules was observed (Figure 4, lane 4).

Cell surface appearance of NEP in PCT cells grown in Petri dishes

To study the kinetics of NEP transport to the apical domain, cells grown in plastic Petri dishes exposing only antigens in the apical domain [20] were pulse-labelled and immunoprecipitated as described previously. As shown in Figure 5, antibodies added to the cell surface after the pulse failed to detect newly synthesized NEP. The molecules were accessible to antibodies only at the 30 min pulse and reached maximum surface expression after the 60 min pulse. Thus at zero time, newly synthesized molecules had not reached the cell surface. These results confirm that, under the conditions of immunoprecipitation, only the cell surface was accessible to the antibodies. NEP molecules recovered by this procedure are probably located mainly on the apical surface, as ultrastructural and binding studies revealed no labelling antigens on the basolateral surface.

DISCUSSION

In this work, we investigated the biosynthesis and targeting of NEP in PCT cells for two reasons: (i) to assess its biogenic pathway in kidney proximal tubules; and (ii) to validate PCT cells in primary culture as a functional system for the study of kidney brush-border-membrane hydrolases.

We demonstrated by immunocytochemical and biochemical approaches that PCT cells cultured in plastic Petri dishes exhibit structural and morphological polarity. The criteria used to validate the development of both morphological and functional polarities have been determined mainly by studies of established epithelial cell lines such as MDCK cells [21,22]. In applying these indices of polarity to the present work, we observed a similarity between primary cultures and this established cell line. Common to both was: (i) the appearance of ultrastructural features characteristic of polarized epithelial cells, i.e. junctional complexes, desmosomes, apical microvilli; and (ii) the fact that, in primary as well as in MDCK cells, NEP showed a similar biogenic pathway.

Indeed, our results revealed that NEP maturation in PCT cells was similar to that described *in vivo* [9] and in MDCK cells [8]. The kinetics of its transport to the plasma membrane were studied by pulse-chase labelling of cells grown in Petri dishes. After a 20 min pulse, NEP appeared as a high-mannose glycosylated form, which prevailed for 15 min and, thereafter,

conversion into the complex glycosylated form took place. The appearance of the mature enzyme in the apical membrane occurred shortly after this conversion, i.e. at 30 min chase. Maximal cell surface expression was reached after a 60 min chase.

The polarized distribution of NEP was demonstrated by culturing PCT cells on permeable supports. We previously reported that PCT cells grow and differentiate on permeable supports [6], forming monolayers that exhibit a pattern of functional differentiation comparable with Petri-dish-grown cells. Considering the absence of colloidal gold-labelling and radiolabelling at the steady-state on the basolateral domain, we can speculate that the major part of the synthesized NEP molecule is directly targeted to the apical domain in PCT cells.

Recent studies describing the conversion of rabbit PCT into a cell monolayer *in vitro* concluded that in spite of the positive physiological response usually obtained with cultured cells, *in vitro* models resemble immature and somewhat modified anatomical models [23]. It is true that PCT cells in culture neither form a brush border as at the apical surface of *in situ* cells nor develop a basal lamina that might allow solid attachment to the substrate. We demonstrated, however, that these cells at 7 or 10 days in culture in a defined medium bore numerous morphological resemblances to kidney proximal cells. It is known that the expression of apical proteins in their specific domain requires a high degree of cell differentiation and close cell interactions. When differentiation is decreased by culture conditions [24] or intracellular contacts are reduced [25], these proteins are not expressed or only weakly expressed in the apical domain because of rapid degradation or sequestration in a vacuolar apical compartment. Moreover, the expression and characteristic targeting phenotype of a particular cell type may also be regulated temporally. A large fraction of dipeptidyl peptidase IV uses the transcytotic route in FRT cells after 1 day of culture but this pathway is replaced by direct apical delivery after several days of culture [26]. As the newly synthesized NEP molecules are efficiently transported and targeted to the cell surface, we can conclude that PCTs are fully differentiated in our culture conditions.

Studies of endogenous protein sorting have produced conflicting results. In MDCK cells, basolateral ($\text{Na}^+ + \text{K}^+$)-ATPase and a number of apical glycoproteins were targeted directly to their appropriate membrane domains [8,27]. In contrast, the polymeric immunoglobulin receptor in MDCK cells and several apical proteins in hepatocytes were targeted first to the basolateral domain before being rerouted to the apical membrane by transcytosis [1,28,29]. Caco-2 cells have an intermediate sorting phenotype with apical proteins using both direct and indirect pathways, and basolateral proteins following only a direct pathway during biogenesis [30]. Whatever the sorting route, it appears that the biogenic pathway of membrane glycoproteins is cell specific and that sorting of apical proteins may be different in the same cell. To determine whether the different results observed with tissues after subcellular fractionation and with virally infected MDCK cells are explained by differences in methodology or inherent differences in the system examined, it is necessary to characterize the biogenesis of endogenous proteins in epithelial cell lines of diverse tissue origin. By scrutinizing the behaviour of particular endogenous components in primary cultures of epithelial cells, it should be possible to determine to what extent established cell lines (MDCK, Caco-2) reflect normal transport processes versus those specific to viral infection, transfection or malignancy.

This study of the biosynthetic pathway of microvillar NEP in PCT cells is the first dynamic approach to test the functional

efficiency of this model. None of the previous studies investigated sorting pathways in primary cells of true epithelial origin. Our experiments clearly indicate that the biogenic pathway of exogenous microvillar membrane hydrolases, such as NEP in the MDCK cell line, is a reflection of normal processes in the kidney. Recent results in our laboratory have shown that the expression of NEP differs markedly from the other marker of intestinal cell differentiation in Caco-2 cells [31]. All the cells synthesize NEP but only a small proportion of them express the protein at the cell surface. Whether this difference is due to the malignant origin of these cells or to a differential processing of NEP in enterocytes has not been elucidated.

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