Polarized distribution of neutral endopeptidase 24.11 at the cell surface of cultured human intestinal epithelial Caco-2 cells

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The human colon cancer cell line Caco-2 undergoes spontaneous enterocytic differentiation during growth, and expresses a number of brush-border-membrane-associated hydrolases typical of a differentiated phenotype. Among these are alkaline phosphatase, dipeptidyl peptidase IV and sucrase-isomaltase (sucrase, EC 3.2.1.48). Neutral endopeptidase 24.11 [EC 3.4.24.11, neprilysin (NEP)] is another abundant protease of normal enterocytes but its presence in Caco-2 cells has not been fully documented yet. In this paper, we show that Caco-2 cell extracts hydrolyse tritiated [D-Ala²Leu⁵]enkephalin with a K_m of 180 μ M, very close to the value obtained for the NEP present in the rabbit kidney (118 μ M). Western-blot analysis of brush-border membranes purified from post-confluent cells revealed a protein with an apparent molecular mass of 94000 Da similar to that of the rabbit kidney NEP. The amount of enzyme in cell extracts increased as a function of the age of the culture, indicating that NEP expression is correlated with the degree of cell differentiation as is also the case for sucrase and dipeptidylpeptidase IV (DPP-IV). Binding of a radiolabelled antibody to Caco-2 cell monolayers grown on semi-permeable filters indicated that 95% of NEP molecules present at the cell surface are on the apical side. Immunocytochemical and flow cytometric analysis of intact and permeabilized cells were also used to investigate the presence of NEP and DPP-IV at the surface of Caco-2 cells. Whereas DPP-IV staining appeared to be homogeneous throughout the entire cell population, NEP-related fluorescence exhibited a bimodal distribution which indicates an uneven expression of the protein at the cell surface. Permeabilization of monolayers with saponin before staining restored a labelling pattern for NEP similar to the one obtained for DPP-IV. This suggests that although DPP-IV and NEP follow similar patterns of expression when enzymic activities are measured on whole-cell extracts, targeting of these brush-border proteins to the cell surface appears to be regulated in different ways.

INTRODUCTION

Neutral endopeptidase (NEP) (neprilysin; EC 3.4.24.11) is a 94 kDa membrane-bound Zn-metalloprotease located at the plasma membrane of many tissues (Kenny, 1986). NEP is particularly abundant in the brush-border membranes of kidney and intestine. The enzyme hydrolyses peptide bonds involving the amino group of hydrophobic amino-acid residues (Matsas *et al.*, 1984) and its best substrates appear to be small peptides rather than proteins. The enzyme has its active site exposed at the cell surface (Kenny & Maroux, 1982) where it is believed to hydrolyse a variety of peptides. In mammalian brain, NEP has been shown to be involved in the inactivation of the opioid peptides [Met]- and [Leu]-enkephalins (Malfroy *et al.*, 1978; Almenoff *et al.*, 1981) and for this reason is sometimes referred to as enkephalinase.

In polarized cells, newly made membrane proteins destined for both cell surfaces travel together from the rough endoplasmic reticulum through the Golgi and the trans-Golgi network (TGN) (Simons & Wandinger-Ness, 1990). Delivery of basolateral membrane components occurs directly from the TGN to that surface in all epithelial cell types examined. In contrast, delivery to the apical surface varies according to the cell type. In Madin–Darby canine kidney (MDCK) cells apically destined molecules such as aminopeptidase N (APN) (Wessels *et al.*, 1990), dipeptidylpeptidase IV (DPP-IV) (Casanova *et al.*, 1991) and

NEP (Jalal *et al.*, 1991) are sorted in the TGN into vesicles and travel directly to the apical surface, while in Caco-2 cells APN and DPP-IV are first delivered to the basolateral surface before reaching the apical surface by transcytosis (Le Bivic *et al.*, 1990a). Very little is known about the expression and the sorting mechanism of NEP in the Caco-2 cell line. Recently, NEP has been found to be expressed at low levels in Caco-2 cells (Howell *et al.*, 1992). In this paper, we show that the enzyme from Caco-2 cells has enzymic and biochemical properties very similar to those of rabbit kidney NEP and that its expression increases in parallel with that of sucrase and DPP-IV as a function of time in culture. We also provide evidence for the polarized cell-surface expression of NEP at the apical surface of filter-grown Caco-2 cells. It appeared in this study that Caco-2 cells exhibit a striking heterogeneity of NEP expression compared with that of DPP-IV. While DPP-IV is homogeneously distributed at the cell surface, NEP displays a mosaic distribution. Homogeneous distribution was restored after permeabilization of the cells with low concentrations of saponin, suggesting the presence of an intracellular storage compartment for NEP.

MATERIALS AND METHODS

Reagents

Cell-culture reagents were purchased from Gibco Laboratories (Grand Island, NY, U.S.A.). Affinity-purified fluorescein-

Abbreviations used: NEP, neutral endopeptidase 24.11 (EC 3.4.24.11); DPP-IV, dipeptidylpeptidase IV; TGN, trans-Golgi network; APN, aminopeptidase N; DMEM, Dulbecco's modified Eagle's medium; TBS, Tris-buffered saline; endo F, endo- β -N-glucosaminidase F; endo H, endo- β -N-glucosaminidase H; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; MDCK, Madin–Darby canine kidney; MDCK-NEP, MDCK cells expressing NEP.

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conjugated rabbit anti-(mouse Ig) antibodies were purchased from Dimension Laboratories (Ontario, Canada). Sheep anti-(mouse Ig) ^{125}I -labelled species-specific F(ab)_2 fragments were from Amersham International (Amersham, Bucks., U.K.). Phosphoramidon and 1-*O*- α -octyl- β -D-glucopyranoside (octyl glucoside) were from Sigma.

Cell culture

The human intestinal cell line Caco-2 was obtained from J. Fogh (Sloan-Kettering Institute for Cancer Research, Rye, N.Y., U.S.A.) in 1986 and cells used in the present studies were at the eightieth passage. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% (v/v) fetal bovine serum, 1% (w/v) non-essential amino acids, 1% (w/v) penicillin and 1% (w/v) streptomycin, and were cultured in humidified incubators at 37 °C in an atmosphere of 8% CO_2 in air. For binding experiments, cells were plated on collagen-coated filters in Transwell culture chambers (Costar Inc., Cambridge, MA, U.S.A.) and grown for 2 weeks after reaching confluence to ensure optimal differentiation (Pinto *et al.*, 1983). The transepithelial electrical resistance was then measured with a Millicell ERS system (Millipore Corp., Bedford, MA, U.S.A.) and found to be in the range of 200–500 ohms/cm². Enzyme activity and cell-flow cytometry were performed on cells grown on plastic Petri dishes for 1–2 weeks after reaching confluence.

Antibodies

Monoclonal anti-(human NEP) antibody (44CD10) (Quackenbush & Letarte, 1985) was a gift from Dr. M. Letarte (Hospital for Sick Children, Toronto, Ontario). Anti-(rabbit NEP) monoclonal antibody (18B5) was derived in our laboratory by immunization of Balb/C mice with purified rabbit NEP. Monoclonal anti-(human DPP IV) (Gorvel *et al.*, 1991) was a gift from Dr. S. Maroux. Coupling of anti-(guinea-pig Ig) and anti-(mouse Ig) to 8 nm colloidal particles was done according to published procedures (Frens, 1973; Roth, 1985; De Mey *et al.*, 1981). ^{125}I -labelled anti-[mouse F(ab)_2] Ig was obtained from Amersham. Biotinylated anti-(mouse Ig) antibodies were from Vectastain Laboratories (Burlington, CA, U.S.A.).

Protein solubilization

Proteins were solubilized from cells with 1% (w/v) octyl glucoside in Tris-buffered saline (TBS) (pH 7.4) as described previously (Devault *et al.*, 1988); solubilization was performed at a cell concentration of 4×10^7 cells/ml. Protein content was determined with the Bradford assay using Bio-Rad reagents and BSA as a standard.

Preparation of cell homogenates

Cells were grown in Petri dishes for up to 21 days. Every 2 days, the culture plates were washed with 2 ml of ice-cold TBS, carefully scraped with a rubber policeman and the resulting cells were homogenized in 600 μl of cold TBS with a Sorvall Omni-Mixer at maximum speed for 1 min. The samples were then used for enzyme and protein assays.

Assay of enzymic activities

NEP activity in solubilized Caco-2 cells or rabbit kidney proximal tubules (Blais *et al.*, 1992) was assayed by monitoring the degradation of the tritiated substrate [Tyr^1 -3,5- ^3H][D-Ala²,

Leu⁵]enkephalin (50 Ci/mmol; Commissariat à l'Énergie Atomique, Saclay, France). Degradation products were separated from intact substrate on Sep-Pak C18 cartridges (Waters) as previously described (Crine *et al.*, 1985). The K_m values were determined by the isotopic dilution method with unlabelled substrate (Peninsula Laboratories, Belmont, CA, U.S.A.); calculations were done with the microcomputer program ENZFITTER (Elsevier-Biosoft, Cambridge, U.K.). Control samples were incubated in the presence of 1 μM -phosphoramidon, a potent NEP inhibitor. All samples were pre-incubated for 15 min in the presence of 10 μM -bestatin and 1 μM -captopril to inhibit aminopeptidase and angiotensin-converting enzyme respectively. To determine the nature of the metabolites obtained after incubating the [D-Ala², Leu⁵]enkephalin substrate with Caco-2 cell solubilizates, samples were analysed by reversed-phase h.p.l.c. on a C18 μ Bondapak column (Waters) (Devault *et al.*, 1988).

Sucrase (EC 3.2.1.48) activities were determined spectrophotometrically according to the method of Kunst *et al.* (1974). DPP-IV activities were also determined spectrophotometrically according to Kojima *et al.* (1980).

Brush-border (apical) membrane purification

Brush-border membranes were purified from Caco-2 cells homogenates as described previously (Blais *et al.*, 1987) using 18 mM- CaCl_2 for non-apical-membrane precipitation (Rousset *et al.*, 1985). When sucrase activity was used as a specific enzyme marker for apical membranes, enrichment factors of 15–17-fold over the starting homogenate were routinely obtained by this technique (Blais *et al.*, 1987).

Endoglycosidase digestions and immunoblotting

Brush-border membranes (20 μg) purified from Caco-2 cells grown on Petri dishes for 26 days were solubilized in TBS containing octyl glucoside. Endoglycosidase digestions were performed by adding directly 0.5 unit of either endo- β -N-glucosaminidase F (endo F) or endo- β -N-glucosaminidase H (endo H) to 50 μl of the protein solution. Incubations were for 16 h at 37 °C. Proteins were then resolved by electrophoresis in 7.5% polyacrylamide slab gels in the presence of SDS (Laemmli, 1970) and electrophoretically transferred to nitrocellulose filters (Schleicher & Schuell, Keene, U.S.A.; pore size 0.45 μm) using published procedures (Towbin *et al.*, 1979). The blots were incubated first with a monoclonal antibody (13 $\mu\text{g}/\text{ml}$) raised against rabbit kidney NEP and then with a biotinylated anti-(mouse Ig) antibody. The position of the bands was visualized using the Vectastain ABC-immunoperoxidase kit from Vector Laboratories.

Indirect immunofluorescence

Post-confluent Caco-2 cells (19 days) grown in Petri dishes were quickly washed in phosphate-buffered saline (PBS) and fixed for 1 h at 4 °C with 4% (v/v) paraformaldehyde in 0.1 M-Sorensen buffer, pH 7.4, ($\text{Na}_2\text{HPO}_4 \cdot 7 \text{H}_2\text{O}/\text{KH}_2\text{PO}_4$). The cells were then incubated for 30 min at room temperature with 50 mM- NH_4Cl in PBS to quench the free-aldehyde groups and 30 min in PBS containing 0.2% gelatin before staining. Incubation with the antibody 44CD10 (12.5 μl of ascites fluid in 1 ml of buffer) was performed at room temperature. Anti-villin monoclonal antibody (a generous gift of Dr. Daniel Louvard, Institut Pasteur, Paris, France) diluted 1:200 was used to assess the epithelial phenotype. For intracellular staining, cells were permeabilized with 0.005% saponin for 30 min. After washing, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-(mouse Ig) for 30 min. The coverslips mounted in

50 % (w/v) glycerol in PBS were examined by epifluorescence microscopy.

Flow-cytometric analysis

A Caco-2 cell suspension containing $(1-2) \times 10^6$ cells was incubated with an excess of 44CD10 monoclonal antibody or anti-DPP IV monoclonal antibody for 45 min in TBS/EDTA (50 mM), rinsed three times and incubated for 45 min with FITC-conjugated rabbit anti-(mouse Ig) antibody (dilution 1:40). MDCK cells expressing recombinant NEP (Jalal *et al.*, 1991) were analysed in parallel using a specific anti-(rabbit NEP) polyclonal antibody (Lemay *et al.*, 1990). For intracellular staining, cells were permeabilized with 0.01 % saponin for 30 min before incubation with antibodies. After three washes, the cells were filtered and analysed by flow cytofluorimetry with an FACS II (Becton Dickinson, Mountain View, CA, U.S.A.). Background fluorescence was corrected using cells that had been incubated with the second antibody only. For fluorescence analysis, the logarithmic scale of the fluorescence channel was converted into arbitrary units.

Distribution of NEP at the cell surface

Tight cell monolayers grown in Transwell culture chambers were incubated at 4 °C for 2 h with 44CD10 monoclonal antibody added to either the apical or the basolateral chamber at dilutions of 1:80 or 1:62 in PBS containing 2 % BSA respectively. After three washing steps, the apical or the basolateral chambers were incubated in the same buffer at 4 °C in the presence of ¹²⁵I-labelled Fab fragments specific for mouse immunoglobulins (specific activity 19–74 Bq/mmol, 500–2000 Ci/mmol) (Amersham) added either to the apical or the basolateral compartment at dilutions of 1:100 and 1:80 respectively. Control filters were probed with only the anti-mouse ¹²⁵I-labelled Fab fragment. The cells were then rinsed twice with PBS, scraped off the filters and solubilized in TBS/octyl glucoside. Protein content was resolved by SDS/PAGE as described above and the autoradiography of the dried gel scanned with an enhanced laser densitometer (LKB Ultrascan XL) using a GELSCAN XL program.

RESULTS

Characterization of NEP activity in Caco-2 cells

Solubilized extracts of Caco-2 cells contained a phosphoramidon-sensitive activity cleaving the Gly-3–Phe-4 bond of tritiated [D-Ala²,Leu]enkephalin characteristic of NEP (Malfroy *et al.*, 1978; Almenoff *et al.*, 1981). Kinetic studies were then performed to compare the properties of the NEP-like activity expressed in Caco-2 cells with those of the kidney NEP (Table 1). A *K_m* value of 180 μM was found for the enzyme expressed by Caco-2 cells, which is close to the value of 118 μM found for the enzyme of kidney proximal tubules. Our results also indicate that NEP specific activity is about 50–100-fold less in Caco-2 cells than in rabbit kidney proximal tubules.

NEP from Caco-2 cells was further characterized by SDS/PAGE and immunoblotting using a monoclonal antibody against rabbit NEP. Purified brush-border membranes from post-confluent cells were solubilized in TBS/octyl glucoside and digested with either endo F or endo H. As shown in Fig. 1, the Caco-2 enzyme migrates with the same electrophoretic mobility as rabbit kidney NEP (lane 1). The protein is sensitive to endo F (Fig. 1, lane 2) but resistant to endo H treatment (Fig. 1, lane 3) indicating the presence of complex sugars.

Enzymic activities of NEP, sucrase and DPP-IV as a function of time in culture

The cell differentiation characteristics of the Caco-2 cells grown in Petri dishes have been extensively studied using the

Table 1. Comparison of NEP enzymic activity in Caco-2 cells and in rabbit kidney proximal tubules

	Specific activity (pmol/min per μg of protein)	<i>K_m</i> (μM)	<i>V_{max}</i> (min ⁻¹)
Tubules	60 ± 12	118 ± 21	273 ± 16
Caco-2 cells	2.51 ± 0.75	180 ± 14	75 ± 2

Fig. 1. Immunoblotting of NEP in Caco-2 cell extracts

Extracts of solubilized Caco-2 cells (100 μg) were analysed by SDS/PAGE and immunoblotting as described in the Material and methods section. Lane 1, untreated sample; lane 2, sample digested with endo F; lane 3, sample digested with endo H. The position of purified kidney proximal tubules NEP (3–5 μg) analysed in parallel on the same gel is also indicated.

Fig. 2. Growth-related differentiation of NEP, sucrase and DPP-IV activities in Caco-2 cells

NEP (a) and sucrase and DPP-IV activities (b) were measured as a function of time in culture. For NEP, each point represents the mean ± s.d. of three determinations as indicated over each bar. Sucrase and DPP-IV activities have been determined in duplicate.

Fig. 3. Polarized distribution of NEP at the cell surface of Caco-2 cells

Caco-2 cells were grown to confluence in 24 mm Transwell chambers, chilled to 4 °C, rinsed with cold PBS and incubated in PBS/BSA for 1 h at 4 °C with an appropriate dilution of monoclonal antibody raised against CALLA (human NEP). The antibody solution was added either to the apical (lane A) or the basolateral (lane B) compartment of the filter unit. After the incubation both sides of the monolayers were washed and incubated for 1 h with anti-(mouse Ig) 125 I-labelled F(ab) $_2$ fragments. The cells were then rinsed twice with PBS, scraped from the filters and analysed by electrophoresis. Control monolayers (CT) were incubated in the same conditions with the anti-(mouse Ig) 125 I-labelled F(ab) $_2$ fragments only. Dried gels were prepared for autoradiography.

different enzyme markers of the brush-border membrane (Jumarie & Malo, 1991). Caco-2 cells begin to differentiate after confluence (day 9). The enzyme activities of sucrase, alkaline phosphatase, and γ -glutamyltranspeptidase increase as a function of the cell culture time to reach a maximal level at day 18. Some activities such as those of DPP-IV remain stable or increase whereas others (sucrase, γ -glutamyltranspeptidase, alkaline phosphatase) may decrease. In this study, we have followed NEP, DPP-IV and sucrase activities as a function of time in culture. As shown in Fig. 2, the sucrase activity (Fig. 2b) increases after day 8 to reach a maximum level by day 14 and decreases after 19 days in culture. NEP and DPP-IV activities showed a different pattern. NEP and DPP-IV activities (Figs. 2a and 2b) also increase after day 8 but do not decrease after day 19. These observations are consistent with the results of previous reports (Blais *et al.*, 1987; Jumarie & Malo, 1991; Howell *et al.*, 1992).

Polarized distribution of NEP at the cell surface of Caco-2 cells

Caco-2 cells were initially grown to confluence on collagen-coated Transwell filters. The surface localization of NEP in these cells was first assessed by measuring the binding of radioactive antibodies to the cell surface. Tight monolayers were incubated with a monoclonal antibody raised against human NEP (44CD10) added either to the apical or to the basolateral domain. The cells were washed and further incubated with 125 I-labelled anti-mouse F(ab) $_2$ fragment. As a control, monolayers were labelled both apically and basolaterally only with the iodinated second antibody. Total cellular extracts were fractionated by gel electrophoresis and radioactive Fab fragments visualized by autoradiography (Fig. 3). While the control cell monolayers (Fig. 3, lane CT) and the basolateral surface (Fig. 3, lane B) did not bind any activity, a strong radioactivity was observed when 44CD10 antibodies were added to the apical surface (Fig. 3, lane A). Quantitative analysis of the autoradiogram with a scanning

Fig. 4. Immunocytochemical study of Caco-2 cell monolayers

Caco-2 cells grown in plastic Petri dishes for 15–19 days were prepared for immunofluorescent staining as described in the Material and methods section. Cells were examined by epifluorescence microscopy at a magnification of 100 $\times$. (a) Intact Caco-2 cells labelled for NEP. (b) Cells permeabilized with 0.005% saponin and labelled for NEP. (c) Cells permeabilized with 0.005% saponin and labelled for villin.

densitometer allowed us to conclude that more than 95% of NEP which was present on the cell surface is in the apical membrane.

Immunocytochemical study of Caco-2 cells

It has been shown that post-confluent Caco-2 cells display a remarkable heterogeneity in their differentiated characteristics. This is particularly striking in the case of sucrase. Immunofluorescent staining with anti-sucrase monoclonal and polyclonal antibodies has consistently revealed the presence of strongly positive and negative neighbouring cells (Pinto *et al.*, 1983; Hauri *et al.*, 1985; Stieger *et al.*, 1988). In contrast, the same cells appeared to be homogeneously stained with antibodies specific for DPP-IV and crypt-cell antigen (Quaroni, 1985). To determine further the extent of NEP distribution in the Caco-2 cell line, cell monolayers were analysed for indirect immunofluorescence using the 44CD10 monoclonal antibody.

Fig. 5. Analysis of NEP expression in Caco-2 cells by flow cytometry

A Caco-2 cell suspension containing $(1-2) \times 10^6$ cells was incubated with either 44CD10 monoclonal antibody for human NEP (a) or with an antibody for DPP-IV (b) and then with FITC-conjugated rabbit anti-(mouse IgG) antibody. For comparison purposes MDCK cells expressing recombinant NEP were analysed in parallel using a specific anti-(rabbit NEP) polyclonal antibody (c). Cells were either stained intact (black area) or after permeabilization with 0.01 % saponin (white area). The cells were filtered and analysed by flow cytofluorometry. Histograms are represented with background staining subtracted.

Immunofluorescent staining of confluent monolayers for NEP demonstrated a marked heterogeneity similar to that observed for sucrase. The staining appeared as small punctates of fluorescence characteristic of an apical localization (Fig. 4a). When confluent monolayers of Caco-2 cells were permeabilized with saponin before fluorescent staining, the whole cell population was found to be labelled with anti-(NEP) antibodies (Fig. 4b). The staining was either perinuclear in some cells or near the plasma membrane in others, but had the same intensity in all cells.

To assess the enterocyte-like phenotype of all post-confluent Caco-2 cells analysed, we used villin, a cytoskeleton-associated protein strongly expressed in differentiated intestinal cells as a marker (Maunoury *et al.*, 1988). When paraformaldehyde-fixed cells were permeabilized to provide access of the antibody to the protein, we observed that villin was uniformly distributed in the whole cell population (Fig. 4c).

Expression of cell-surface NEP in a sub-population of Caco-2 cells

To confirm and analyse more accurately the mosaic distribution of NEP in Caco-2 cells, suspensions of Caco-2 cells were labelled either with anti-(NEP) antibody (44CD10) or anti-(DPP-IV) antibody and analysed by flow cytometry. As a control, MDCK cells that express rabbit NEP (MDCK-NEP) (Jalal *et al.*, 1991) were also analysed with an anti-(rabbit-NEP) antibody. As shown in Fig. 5(a), the distribution of 44CD10 immunofluorescence in the Caco-2 cell population exhibited a rather broad distribution suggesting an heterogeneous expression of the antigen at the cell surface. However, when the cells were permeabilized with saponin, a single symmetrical peak of fluorescence was observed at a much higher intensity. By contrast, labelling of either intact or permeabilized cells with anti-(DPP-IV) antibody produced a single symmetrical peak of fluorescence intensity, demonstrating a homogeneous cell-surface distribution of DPP-IV (Fig. 5b). To rule out the possibility that the change in fluorescence could be caused by a non-specific effect of saponin on the cell membrane, permeabilization of MDCK-NEP was also performed. This treatment did not produce an increase of fluorescence intensity (Fig. 5c).

DISCUSSION

Our results show that Caco-2 cells contain an enzyme which appears to be very similar to NEP: it cleaves [D-Ala²,Leu]enkephalin at the Gly-3-Phe-4 bond, is inhibited by phosphoramidon and thiorphan, has the same apparent molecular mass on SDS/PAGE, and is recognized by polyclonal and monoclonal antibodies raised against the human or rabbit enzyme. As previously observed for NEP in other epithelial cell types, the expression of this protease in Caco-2 cells was also found to be highly polarized with a predominant apical localization. However, NEP levels in Caco-2 cells were very low as they represented only 1-5 % of those found in the intestine and the kidney.

Enterocytes of the small intestine, like epithelial cells of the kidney proximal tubule, express many microvillar proteins. Even though Caco-2 cells originate from colonic cells, which normally lack a well-developed brush border, they were recently found to express APN, DPP-IV, angiotensin-converting enzyme, alkaline phosphatase, aminopeptidase W, membrane dipeptidase, NEP and γ -glutamyltranspeptidase (Howell *et al.*, 1992). Our results describing the presence of NEP in the Caco-2 cell line are in good agreement with this previous study and are also consistent with the enzyme being synthesized by both fetal and adult colon (Pollard *et al.*, 1991). It is noteworthy, however, that the expression of the enzyme by these cells differs markedly from the other markers of intestinal cell differentiation. In confluent cultures, the specific activity of NEP and DPP-IV increases steadily as a function of time, whereas that of sucrase decreases after day 19. NEP, like DPP-IV and APN, is expressed by the entire intestinal epithelium (Pollard *et al.*, 1991). It may therefore represent a new differentiation marker of intestinal epithelial cells.

Immunofluorescent staining and flow-cytometric analysis of NEP demonstrated a mosaic pattern of expression very different from the homogeneous staining observed in parallel for DPP-IV. Recently, Beaulieu & Quaroni (1991) observed marked differences among various clones of Caco-2 cells in sucrase activity and in immunoreactive cell-surface sucrase, but not in APN and DPP-IV activities. To assess more accurately the cause of the heterogeneous expression of NEP we studied the distribution of the enzyme after permeabilization of the cells with saponin. Under those conditions all cells were found to synthesize NEP as

well as DPP-IV. This indicates that Caco-2 cells that do not express NEP at their surface are not defective in NEP synthesis, but rather accumulate the protein in some intracellular compartments. One explanation for the presence of these different phenotypes in the same population may be that mechanisms which trigger and modulate expression of differentiated function in Caco-2 cells are modulated by local factors or cell-cell interactions.

It was reported that intestinal brush-border enzyme glycoproteins are transported to the microvillar membrane at different rates in the Caco-2 cell line (Stieger *et al.*, 1988) and that proper glycosylation might be essential for efficient post-Golgi transport (Matter *et al.*, 1989). Thus, transport to the site of complex glycosylation is slow for sucrase and fast for DPP-IV. Furthermore two naturally occurring mutants of sucrase with impaired intracellular transport displayed an immature conformation (Naim *et al.*, 1988; Matter & Hauri, 1991). It was then proposed that differential kinetics of folding might be the underlying cause for both pre- and intra-Golgi steps of asynchronous intracellular transport and that a proper tertiary structure might be a prerequisite for sucrase to leave the Golgi apparatus (Matter & Hauri, 1991). The poor cell-surface expression of NEP by an important population of differentiated Caco-2 cells might be due to an improper folding of NEP in these cells. This possibility, however, appears to be unlikely as NEP is still recognized by our monoclonal and polyclonal antibodies after permeabilization of Caco-2 cells with saponin.

In a recent report, immunoelectron microscopy was used to localize sucrase and DPP-IV activity in intracellular compartments identified as small vesicles, multivesicular bodies and lysosomal bodies (Klumperman *et al.*, 1991). Complete disruption of the microtubular network by colchicine and nocodazole in Caco-2 cells also resulted in the appearance of intracellular vacuoles containing apical markers (Gilbert & Rodriguez-Boulant, 1991). Reducing intercellular contacts between MDCK cells also results in the appearance of an intracellular storage compartment for apical membrane proteins (Vega-Salas *et al.*, 1987). It has been proposed that these vacuolar apical compartments may play an as yet unrecognized role in the biogenesis of the apical plasma membrane during the differentiation of normal epithelia. Our results, suggesting the presence of an intracellular pool of NEP in many cells of Caco-2 cultures, are consistent with the existence of such a vacuolar compartment. Whether NEP in this compartment results from endocytosis of molecules after their insertion in the membrane or from blockage of the targeting of newly synthesized proteins remains to be determined.

Earlier publications (Breitfeld *et al.*, 1989; Matter *et al.*, 1990) suggest that epithelial cells may have both transcytotic and direct routes to the apical surface and that a given apical protein may use one or the other or both. MDCK cells use the direct route for all apical proteins studied so far (Caplan *et al.*, 1986; Le Bivic *et al.*, 1990b; Lisanti *et al.*, 1989; Jalal *et al.*, 1991) and hepatocytes only an indirect route (Bartles *et al.*, 1987). Caco-2 displays both transcytotic and direct routes to the apical surface. Some authors concluded that this intestinal epithelial cell line reconciles the two different models of protein sorting of renal and hepatic cells. It would be interesting in this context to determine whether NEP is processed similarly or differently in the Caco-2 cell line than in the MDCK cell line. However, the amount of NEP synthesized by Caco-2 cells is very low. It would be necessary to induce the expression of a recombinant enzyme at higher levels. This approach would hopefully allow identification of the mechanisms controlling the level of NEP at the cell surface and allow investigation of the hypothesis of cell-specific targeting pathways.

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