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Mosaic expression of membrane peptidases by confluent cultures of Caco-2 cells

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The cell-surface expression of endopeptidase-24.11 (EC 3.4.24.11) on Caco-2 cells cultured to confluency is markedly heterogeneous unlike that of dipeptidylpeptidase IV (EC 3.4.14.5). Here we have investigated the cell-surface expression of three other ectopeptidases: angiotensin converting enzyme (EC 3.4.15.1), aminopeptidase N (EC 3.4.11.2) and aminopeptidase W (EC 3.4.11.16). We show by indirect immunofluorescent staining that these three enzymes are present on the surface of some cells but not on others. However, these enzymes were detected in the majority of detergent-permeabilised Caco-2 cells indicating the presence of intracellular pools of these enzymes. This suggests that there may either be differential regulation of apical transport for these peptidases or that they recycle at different rates.

Membrane peptidase; Immunofluorescent staining; Caco-2 cell

1. INTRODUCTION

The Caco-2 cell line was derived from a human adenocarcinoma in 1976 [1] and has since been widely studied (for review see [2]). On reaching confluency Caco-2 cells undergo differentiation into enterocyte-like cells and express many hydrolases on the apical brush border [3–6]. These hydrolases include some disaccharidases, for example sucrase-isomaltase and lactase-phlorizin hydrolase [4], and numerous peptidases [6]. Using indirect immunofluorescent staining, the membrane peptidase dipeptidylpeptidase IV (DPPIV) has previously been shown to be homogeneously distributed on the surface of Caco-2 cells [4,7]. In contrast, we recently reported that another cell-surface peptidase, endopeptidase-24.11 (E-24.11), was present on the surface of some cells but not on others [6]. Jalal et al. [8] have confirmed this observation and also shown that E-24.11 is in fact expressed by all cells but in some is confined to an intracellular pool.

In the present study, the cell-surface expression of five membrane peptidases on confluent Caco-2 cells has been investigated and evidence is presented to show that the mosaic pattern of E-24.11 surface staining is also observed for other cell-surface peptidases. Angiotensin converting enzyme (ACE), aminopeptidase N (AP-N) and aminopeptidase W (AP-W) were also detected only on the surface of some cells, but found to be located in intracellular pools in the majority of the cells examined.

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Abbreviations: ACE, angiotensin converting enzyme; AP-N, aminopeptidase N; AP-W, aminopeptidase W; DPPIV, dipeptidylpeptidase IV; E-24.11, endopeptidase-24.11; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline.

2. EXPERIMENTAL

2.1. *Materials*

Polyclonal antibodies RP161, RP181, RP125 and RH179 were generated in rabbits immunised with porcine E-24.11, DPPIV, AP-W and human ACE, respectively. They were all affinity-purified and shown to be monospecific by immunoblotting. The murine monoclonal antibody 22A5 (specific for human AP-N) was kindly supplied by Dr. M.A. Horton (I.C.R.F., London, UK). Fluoroscein isothiocyanate (FITC)-conjugated anti-rabbit and anti-mouse IgGs were obtained from the Sigma Chemical Co. (Poole, Dorset, UK). Caco-2 cells were kindly donated by Dr. D.M. Swallow (MRC Human Biochemical Genetics Unit, University College, London, UK).

2.2. *Maintenance of Caco-2 cells*

Caco-2 cells were grown to confluency on glass coverslips in six-well plates (Linbro, Flow Laboratories, Rickmansworth, UK) using Dulbecco's minimal essential medium supplemented with 20% heat-inactivated fetal calf serum (both Imperial Laboratories, Hounslow, Middlesex, UK), 1% non-essential amino acids, 100 U/ml penicillin and 0.1 mg/ml streptomycin (all Gibco BRL, Uxbridge, Middlesex, UK) at 37°C in a 5% CO_2/95% air atmosphere with the medium changed 48 h after seeding and daily thereafter [6]. Cells were seeded at 1.2×10^4 cells/cm^2 and used between pasages 93 and 98.

2.3. *Indirect immunofluorescent staining*

After growing cells in culture for 21 days, coverslips were transferred to clean six-well plates and washed gently three times with 2 ml phosphate-buffered saline (PBS) (140 mM NaCl, 10 mM sodium phosphate buffer, pH 7.4) at 37°C. Cells were then fixed with 1 ml of paraformaldehyde (4% w/v in PBS) at 37°C and stood for 20 min at room temperature. Coverslips were washed and those used to examine intracellular antigens were incubated with 2 ml of Triton X-100 (0.1% w/v in PBS) for 4 min at room temperature to permeabilise the cells. Following a 10 min incubation with 2 ml NH_4Cl (50 mM in PBS), the coverslips were washed again and non-specific binding sites blocked

by the addition of 2 ml of gelatin (0.04% w/v in PBS) for 15 min. After washing, coverslips were then placed on a 0.1 ml drop of primary antibody (RP161, RP181, RP125, RH179 or 22A5 diluted 1:500 in PBS gelatin) for 1 h. Donkey anti-rabbit (or anti-mouse for 22A5) FITC-conjugated IgG secondary antibody (diluted 1:50 in PBS gelatin) was centrifuged (8000 × g) before use and 0.1 ml of the supernatant applied to washed coverslips for 30 min at 4°C [6]. Following further washing, the coverslips were mounted on microscope slides and viewed either by epi-illumination using a Zeiss microscope or by Leica scanning confocal laser microscopy.

3. RESULTS AND DISCUSSION

Fig. 1 shows indirect immunofluorescent staining of DPPIV, E-24.11, ACE, AP-N and AP-W on intact Caco-2 cells at day 21. When the primary antibody was replaced with a non-immune serum control, no immunofluorescent staining was observed. As previously reported, staining on intact cells was found to be homogeneous for DPPIV [4,7] and heterogeneous for E-24.11 [6,8]. Indirect immunofluorescent staining of ACE, AP-N and AP-W showed surface staining on some but not all of the cells. However, these latter three enzymes were expressed in the majority of permeabilised cells as indicated in Fig. 2. The proportion of positively staining cells present was quantified using 8 randomly chosen fields of view (Table I). The percentage of surface staining on intact cultures ranged from 22% for AP-N to nearly 100% for DPPIV. Staining in permeabilised cultures was 88% for E-24.11 and more than 90% for the other four peptidases.

Table I

The expression of ACE, AP-N, AP-W, DPPIV and E-24.11 on and in Caco-2 cells

Enzyme	Percentage of staining	
	Intact cells	Permeabilised cells
ACE	77 ± 2	96 ± 1
AP-N	22 ± 6	96 ± 1
AP-W	41 ± 6	92 ± 1
DPPIV	98 ± 0	94 ± 1
E-24.11	36 ± 7	88 ± 2

Immunofluorescently labelled coverslips were viewed by fluorescent microscopy and the number of positively staining cells were counted as a percentage of the field of view. Results are the mean ± SEM. of 8 randomly chosen fields counted in duplicate.

Confirmation of intracellular staining for E-24.11, ACE, AP-N and AP-W was achieved using confocal microscopy. One example is shown (Fig. 3) of Caco-2

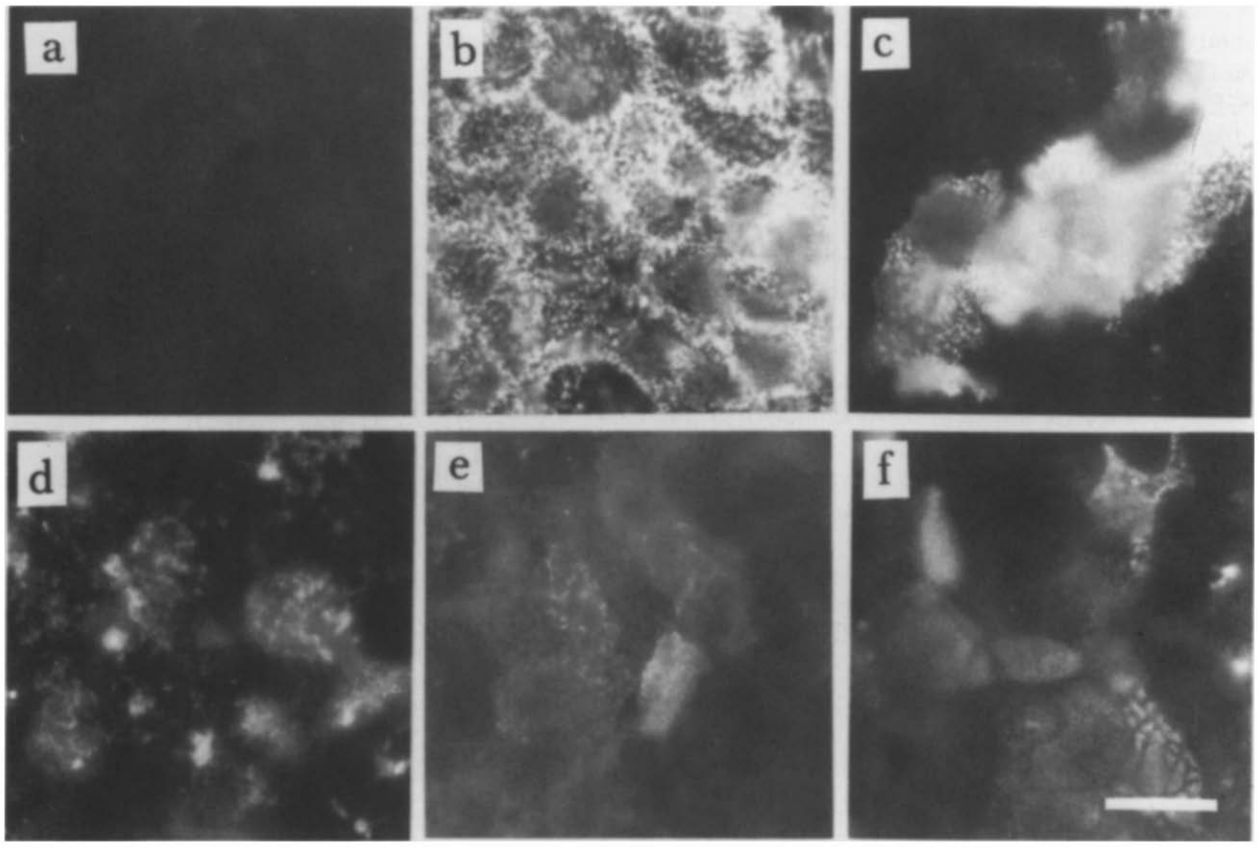

Fig. 1. Indirect immunofluorescent staining of membrane peptidases on intact Caco-2 cells. (a) Control using non-immune serum. (b) DPPIV identified using RP181. (c) E-24.11 identified using RP161. (d) ACE identified using RH179. (e) AP-N identified using 22A5. (f) AP-W identified using RP125. Bar = 25 μm.

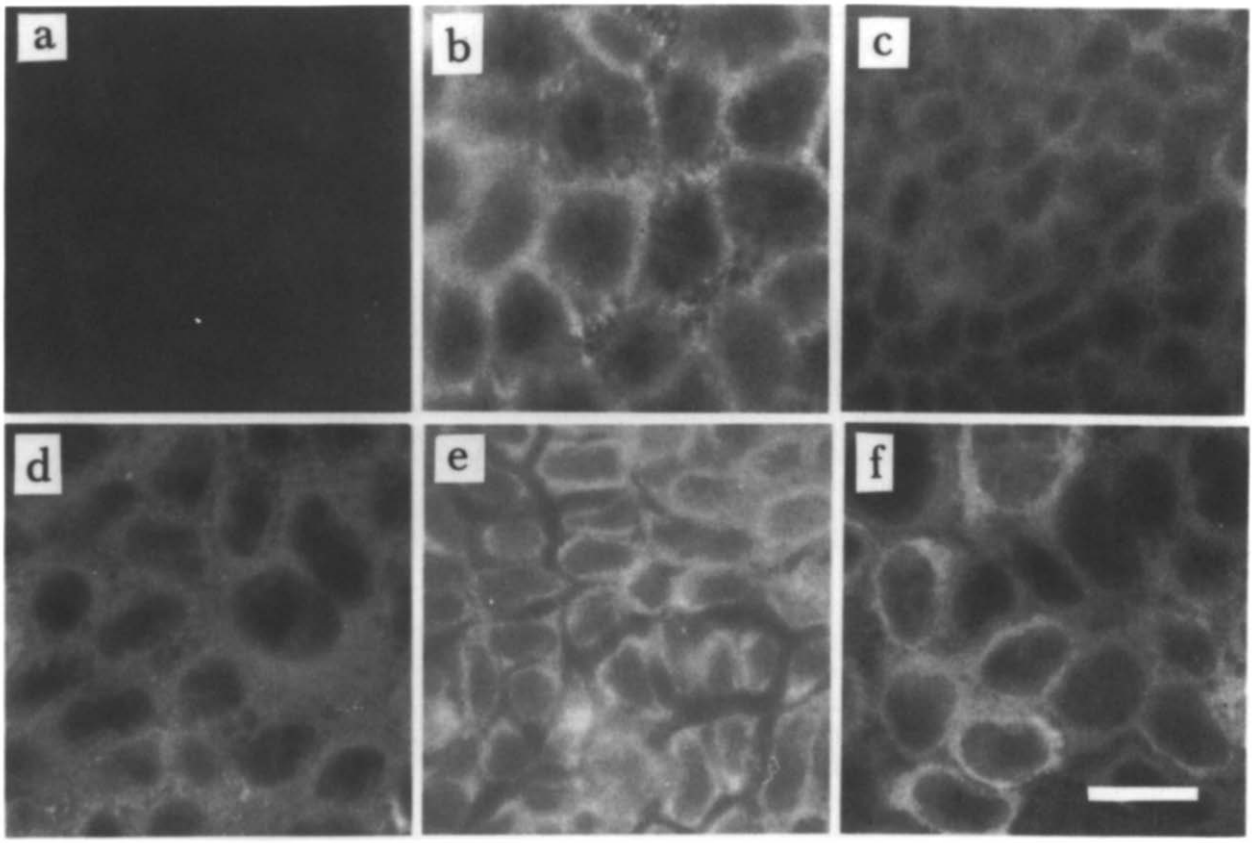

Fig. 2. Indirect immunofluorescent staining of membrane peptidases in Caco-2 cells permeabilised by Triton X-100. (a–f) As detailed in the legend to Fig. 1. Bar = 25 μm.

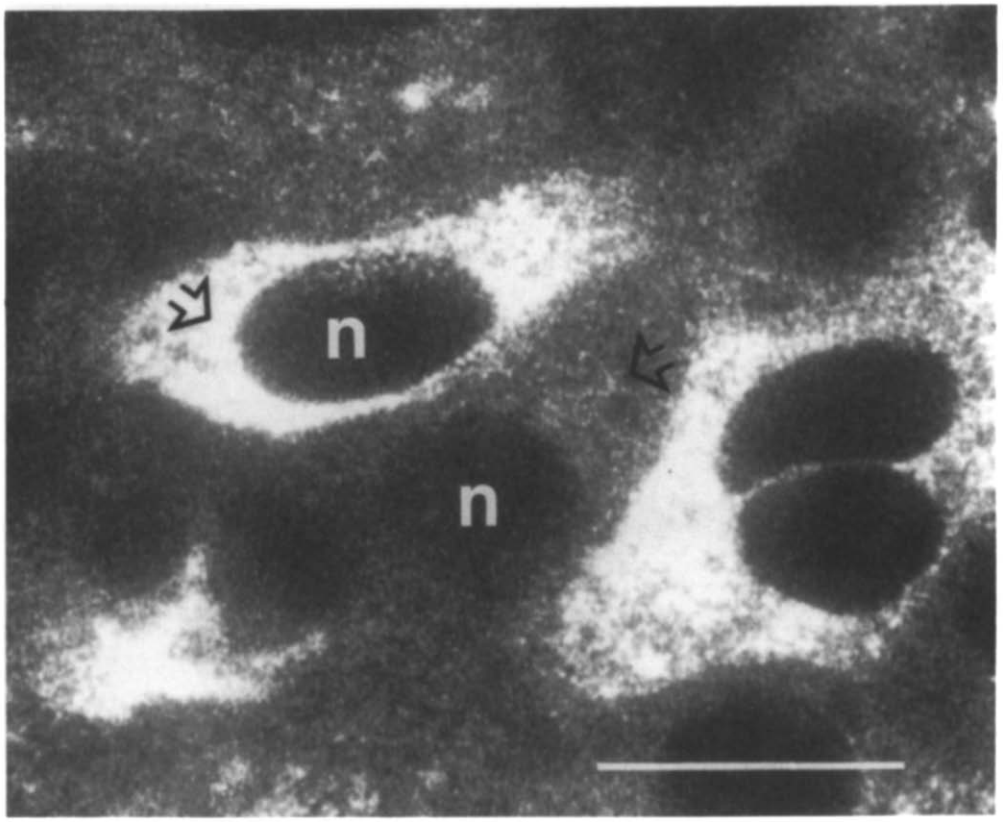

Fig. 3. Scanning confocal laser micrograph of ACE detected by indirect immunofluorescent staining. The computerised image is of a section approximately three-quarters the distance from the apical surface to the base of the cell monolayer. Notice that immunofluorescent staining (arrow) is seen outside of the nuclei (n). Bar = 25 μm.

cells immunofluorescently stained for ACE. This computerised image of a section approximately three-quarters the distance from the apical surface to the base of the cell monolayer reveals intracellular staining in regions around the nuclei.

Many groups have shown that the Caco-2 cell line is heterogeneous with regard to sucrase-isomaltase expression [3,4,9,10]. However, Vachon and Beaulieu [10] recently reported that the heterogeneity seen with sucrase isomaltase immunostaining is a transient phenomenon. We have previously reported that Caco-2 cells showed heterogeneous E-24.11 surface staining [6] and Jalal et al. [8] have since suggested that this may be due to an impaired apical transport system for this enzyme. The results reported here show that there is a marked mosaic expression of many of the membrane peptidases expressed on Caco-2 cells. Some cells have been shown to express ACE, AP-N, AP-W and E-24.11 on their apical surface whereas other cells do not. However, the fact that the majority of the cells do accumulate these enzymes intracellularly suggests either that there are marked differences in the apical transport of these ectoenzymes or that they are recycled at different rates from the surface to the interior. This may have important consequences as Caco-2 cells are increasingly used as a model system for enterocytic differentiation and thus caution should be exercised as this cell line is not as homogeneous, at least at certain stages, as immunostaining for DPPIV would suggest.

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