Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Acta histochem. (Jena) 98, 323–331 (1996)
Gustav Fischer Verlag Jena · Stuttgart · New York

Acta histochemica

Immunoelectron microscopic single and double labelling of aminopeptidase N (CD 13) and dipeptidyl peptidase IV (CD 26)

Thoralf Stange[1], Ursula Kettmann[2] and Hans-Jürgen Holzhausen[3]

[1]Department of Otorhinolaryngology, [2]Department of Physiological Chemistry and [3]Department of Pathology, Faculty of Medicine, University of Halle-Wittenberg, Magdeburger Str. 12, D-06097 Halle, Germany

Accepted 28 April 1996

Summary

Using ultrathin cryosections and immunogold labelling, aminopeptidase N (CD 13) and dipeptidyl peptidase IV (CD 26) were localized on the luminal side of the brush border membrane of proximal tubular cells in human kidney as well as of enterocytes from rat small intestine. Furthermore, both enzymes could be detected on the cell surface of human T lymphocytes and especially aminopeptidase N on human synovial fibroblasts. Gold labelled vesicular structures were also found in the cytoplasm in the apical part of renal proximal tubular cells and synovial fibroblasts. In human kidney the colocalization of the two membrane antigens was possible by using several double labelling methods.

Key words: aminopeptidase N – CD 13 – dipeptidyl peptidase IV – CD 26 – cell surface antigens – immunoelectron microscopy – cryoultramicrotomy – immunogold labelling – double labelling

Introduction

Aminopeptidase N (microsomal alanyl aminopeptidase, mAAP, EC 3.4.11.2, CD 13) and dipeptidyl peptidase IV (DPP IV, EC 3.4.14.5, CD 26) represent membrane exopeptidases, whose biochemical properties and expression sites were intensely investigated. The two membrane proteases are primarily found in the microvillar membrane of renal proximal tubular cells and small intestinal enterocytes of mammals (McDonald and Barrett, 1986), but they are also present in the plasma membranes of various tissues such as liver, parotid and submandibular acinar cells, placenta and blood vessels (Gossrau, 1985). DPP VI and APN can be detected on the cell surface of human synovial fibroblasts (Bathon et al., 1992) and especially APN on synaptic membranes of the central nervous system (Gros et al., 1985). In the immune system DPP VI occurs as a specific marker for T lymphocytes (Mentlein et al., 1984), APN is localized on monocytes, neutrophil granulocytes and in small amounts also on T lymphocytes (Ashmun and Look, 1990; Kunz et al., 1993). Riemann et al. (1993) found an expression of aminopeptidase N on synovial fluid T cells from patients with different forms of joint effusions.

Correspondence to: H.-J. Holzhausen

Both enzymes participate in the final stage of peptide hydrolysis in the renal proximal tubules and small intestine (McDonald and Barrett, 1986) and modify the effect of bioactive peptides like enkephalins (APN), substance P and casomorphins (DPP IV) (De la Baume et al., 1983; Gros et al., 1985; Nausch et al., 1990). The enzymes take also part in the activation and proliferation of lymphocytes (Stein et al., 1989). APN serves as a receptor for coronaviruses, which are RNA viruses that cause respiratory and gastrointestinal diseases (Delmas et al., 1992). DPP IV has coreceptor function for HIV viruses (Callebaut et al., 1993). Both peptidases are involved in the cell adhesion of carcinoma cells in metastatic processes (Johnson et al., 1993; Saiki et al., 1993).

During the last few years several cell surface antigens were differentiated as membrane peptidases. APN corresponds to the cell surface antigen CD 13 (Look et al., 1989) and DPP IV to CD 26 (Ulmer et al., 1990).

Nevertheless many physiological functions of these peptidases are still unknown. In order to receive further informations about the physiological role of membrane proteases it is necessary, to find out their precise cellular location (Gossrau, 1985).

Compared with the light microscopic visualisation of proteases, the electron microscopic demonstration methods for these enzymes have been less frequently applied until now. This limited application of protease ultracytochemistry is explained by the comparatively strong inhibition of the proteases by aldehydes, the co-reaction of proteases different from those under investigation, as well as the reduced stability and moderate electron density of the osmicated final reaction product (Gossrau, 1993).

The detection of APN and DPP IV with immunoelectron microscopical methods has similar problems. Because of their position on the external cell surface it is difficult to find out the extact localization of ectoenzymes such as APN and DPP IV with immunoultrastructural methods (Kettmann et al., 1992 b). The problem with the preembedding method is the difficulty of penetration of markers and antibodies into the cells. Hence intracellular antigens are often not detectable. To improve the limited penetration pre-treatment with detergents is generally necessary, but a too high concentration of detergents can cause membrane damage, and may affect the distribution of the antigen associated with the cell membrane (Mizuno, 1993). In the postembedding method, only those antigens exposed at the surface of resin-embedded sections are immunolabelled. Some antigens, especially membrane proteins, are very labile to fixation, dehydratation, embedding and polymerisation (Mizuno, 1993). Glycosylated enzymes with large extracellular domains bound to membranes via a short hydrophobic peptide such as APN or DPP IV are not labelled, if the tissue is embedded in resins and the results are independent of the mode of dehydration and type of resin. Conventional resin embedding as well as low temperature embedding and freeze-substitution failed to demonstrate the localization of APN by immunogold labelling (Kettmann et al., 1992 b).

Using cryoultramicrotomy in combination with immunogold labelling APN and DPP IV were detectable both on the luminal side of the brush border membrane of proximal tubular cells in rat kidney and intracellularly as potential transport vesicles operating between the Golgi complex and surface membranes (Kettmann et al., 1992 a, b). This method represents a useful compromise between preservation of antigenicity and morphology (Slater, 1993). Immunogold labelling in combination with cryoultramicrotomy has been proven to be a useful method for the visualisation and localization of cell surface and intracellular proteins (Mizuno, 1993). Because of their abundant expression on the microvillus membrane of proximal tubular cells of the kidney and small intestinal enterocytes, these cells are especially suitable for methodological investigations to localize and also colocalize APN and DPP IV, and were therefore used for the present investigation. Since double labelling allows to study the distribution of two antigens within one cryosection and colloidal gold markers are very suitable for double labelling

methods, because gold particles of different size are available, we also used this technique to receive further information on APN and DPP IV at the ultrastructural level.

Materials and Methods

Antisera. Monoclonal antibodies (mouse) (mAB) against CD 13 (clone SJ.1D1) and CD 26 (clone BA5) were purchased from Dianova (Hamburg). Polyclonal monospecific rabbit-anti-rat-(pRAR)APN antiserum (used by Kettmann et al., 1992b) and polyclonal monospecific rabbit-anti-rat-(pRAR)DPP IV antiserum (gift of Dr. A. Heider, Institute of Pathological Biochemistry, Martin Luther University of Halle, Germany) were used for labelling the rat small intestine. For the double labelling investigations polyclonal rabbit-anti-pig-(pRAP)DPP IV antiserum could be used because of its cross-reactivity with human DPP IV. This specificity was proved by 2-dimensional immunoelectrophoresis according to Laurell (1965) and by Western blot described by Kettmann et al. (1992b).

Electron microscopy embedding technique. Tissues. Small pieces of fresh human kidney cortex (operation specimens) and sections of small intestine from rats after sacrifice in ether anesthesia were washed with ice-cold PBS (pH 7.4) and fixed in 2% formaldehyde and 0.02% glutaraldehyde in PBS for 30 min at room temperature, followed by 3 washes with PBS. *Cells.* After centrifugation and washing twice in PBS cultured human synovial fibroblasts and IL-2-stimulated T lymphocytes (gift of Dr. D. Riemann, Institute of Medical Immunology, Martin Luther University of Halle, Germany) were fixed with 2% formaldehyde and 0.02% glutaraldehyde in PBS for 20 min, followed by 2 washes with PBS and subsequently embedded in 7% gelatine (Sigma, St. Louis, USA) in PBS. Afterwards all fixed samples were incubated with 1.15 M sucrose and 10% polyvinylpyrrolidone (PVP K15, Fluka, Buchs, Switzerland) in PBS for approximately 2 days at 5 °C. Further processing and cryosectioning were done following the methods of Tokuyasu (1973, 1989). Ultrathin cryosectioning was performed with a Reichert-Jung FC 4E cryoultramicrotome.

Immunolabelling. The cryosections were transferred to formvar-coated nickel grids (Bio Cell Plano, Marburg), washed with PBS, afterwards with PBS containing 50 mM glycine and finally with 0.5% albumin (Sigma, St. Louis, USA) and 0.2% gelatine (Sigma, St. Louis, USA) in PBS. The sections were incubated in a moist chamber with the specific antiserum (dilution 1 : 100) at 5 °C overnight. The reaction with gold-labelled secondary antibody or protein A was done for 1 h at room temperature. Goat-anti-mouse IgG (GAM, Aurion Immuno Gold, Wageningen, The Netherlands) was labelled with 6 nm and 12 nm gold particles, protein A (Bio Cell Plano, Marburg) with 10 nm gold particles. After washing in PBS and tri-distilled water the sections were embedded in 1.1% tylose (Fluka, Buchs, Switzerland) containing 0.5% uranyl acetate. Controls were done by replacing the specific serum with the respective preimmunserum. *Double labelling.* In the first technique APN and DPP IV were successively labelled with antibodies raised in the same species (Geuze et al., 1981). The first antigen was marked according to the standard labelling procedure with GAM 6 nm gold. The free binding places of the primary antibody were saturated by incubation with unlabelled goat-anti-mouse IgG (Immunpräp., Berlin) 0.01% in PBG for 15 min. The other antigen was labelled with a specific antibody and GAM-conjugated gold particles of different diameter (15 nm, Aurion Immuno Gold, Wageningen, The Netherlands) as used for the first incubation. In the second technique the two antigens were simultaneously labelled with antibodies from different origin (Tapia et al., 1983). The procedure was carried out as common labelling with the mixed primary (pRAP-DPP IV antiserum and mAB against GD 13) and secondary antibodies [GAM-6 nm gold and goat-anti-rabbit IgG (GAR) 10 nm gold (Bio Cell Plano, Marburg)]. To control successfully double labelling one of the two specific antisera was replaced by the respective preimmunserum. Additionally, the labelling sequence of APN and DPP IV was changed. Furthermore, the double labelling results were compared with the corresponding single labelling.

Results

All figures show good preservation of APN and DPP IV antigenicity as well as ultrastructural morphology. APN and DPP IV were found on the luminal side of the brush border membranes of human kidney proximal tubular cells (Fig. 1a, b) and enterocytes of rat small intestine (Fig. 5a, b). A prominent, regular and diffuse labelling of both peptidases was found on these tissues. Control experiments (data not shown) with the respective preimmunserum did not show non-specific labelling.

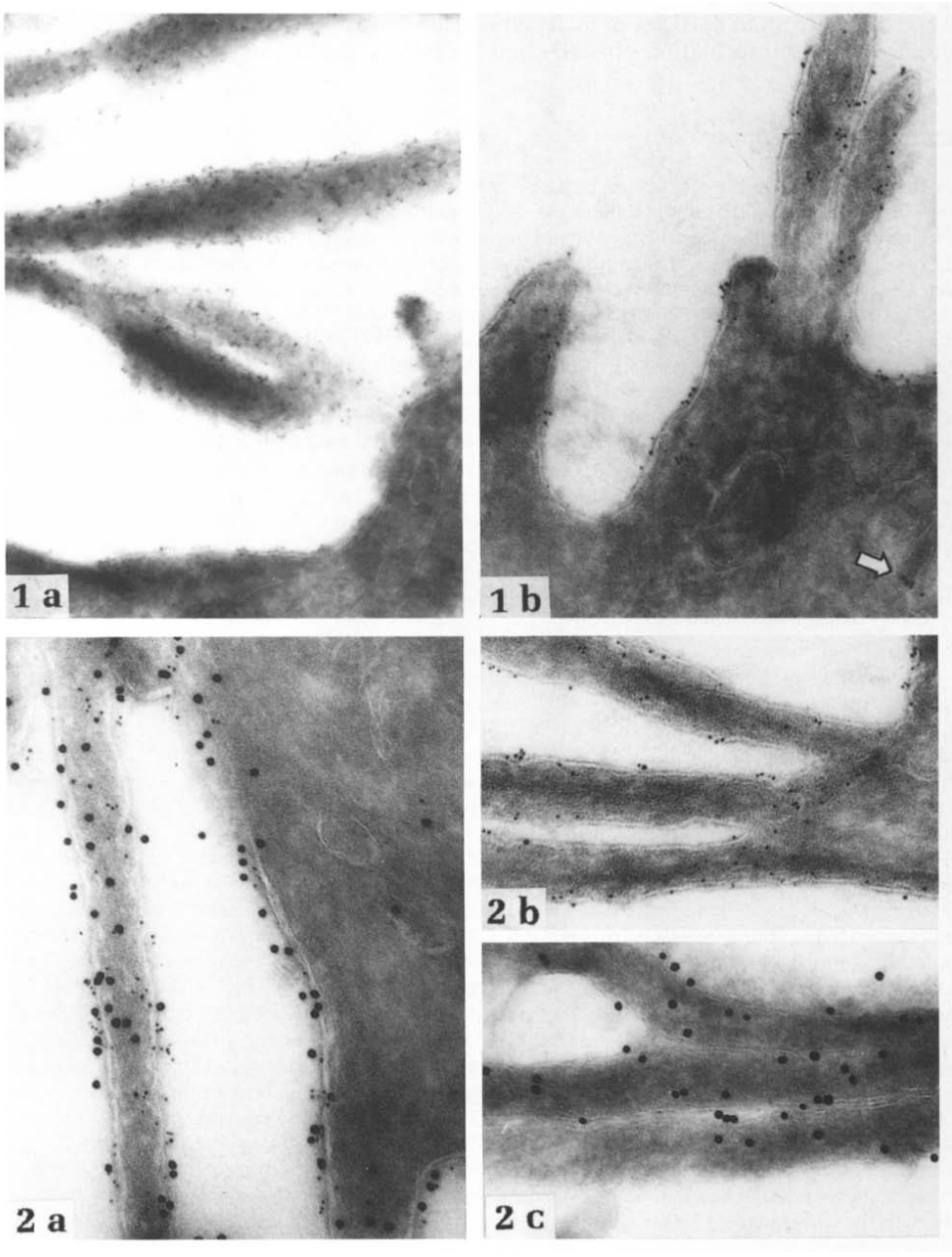

Fig. 1. Single immunolabelling of ultrathin cryosections of brush border from human kidney proximal tubular cells. a. with CD 13-mAB/GAM-6 nm gold b. with CD 26-mAB/GAM-6 nm gold, see intracellular labelling (→). ×80.000. Fig. 2. Successive double labelling of ultrathin cyosections of brush border from human kidney proximal tubular cells. a. with CD 26-mAB/GAM-6 nm gold and CD 13-mAB/GAM-15 nm gold, b. control labelling using preimmunserum/GAM-15 nm gold and CD 26-mAB/GAM-6 nm gold, c. control labelling using preimmunserum/GAM-6 nm gold and CD 13-mAB/GAM-15 nm gold. ×80.000.

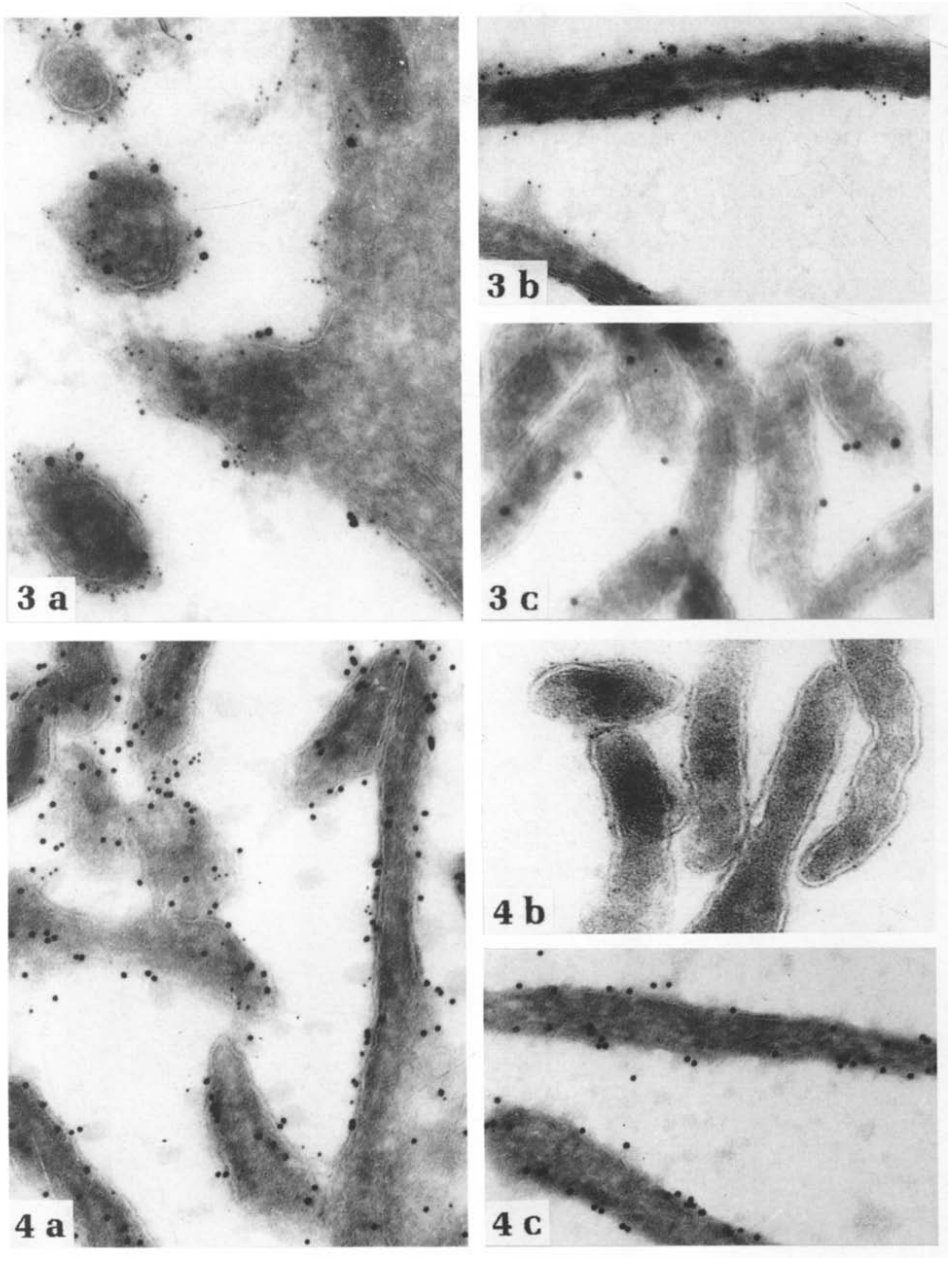

Fig. 3. Successive double labelling of ultrathin cryosections of brush border from human kidney proximal tubular cells. a. with CD 13-mAB/GAM-6 nm gold and CD 26-mAB/GAM-15 nm gold, b. control labelling using preimmunserum/GAM-15 nm gold and CD 13-mAB/GAM-6 nm gold, c. control labelling using preimmunserum/GAM-6 nm gold and CD 26-mAB/GAM-15 nm gold. ×80.000. Fig. 4. Simultaneous double labelling of ultrathin cryosections of brush border from human kidney proximal tubular cells. a. with CD 13-mAB/GAM-6 nm gold and pRAP-DPP IV antiserum/GAR-10 nm gold, b. control labelling using preimmunserum/GAR-10 nm gold and CD 13-mAB/GAM-6 nm gold, c. control labelling using preimmunserum/GAM-6 nm gold and pRAP-DPP IV antiserum/GAR-10 nm gold. ×80.000.

The double labelling experiments showed a close spatial relationship between APN and DPP IV (Figs. 2a, 3a, 4a). Using the successive or simultaneous method no substantial differences in double labelling were seen. No cross-reactivity occurred in double labelling because control experiments led to identical results (Figs. 2b, c, 3b, c, 4b, c). Labelling density for APN appeared to be higher than for DPP IV (see also Kenny and Maroux, 1982).

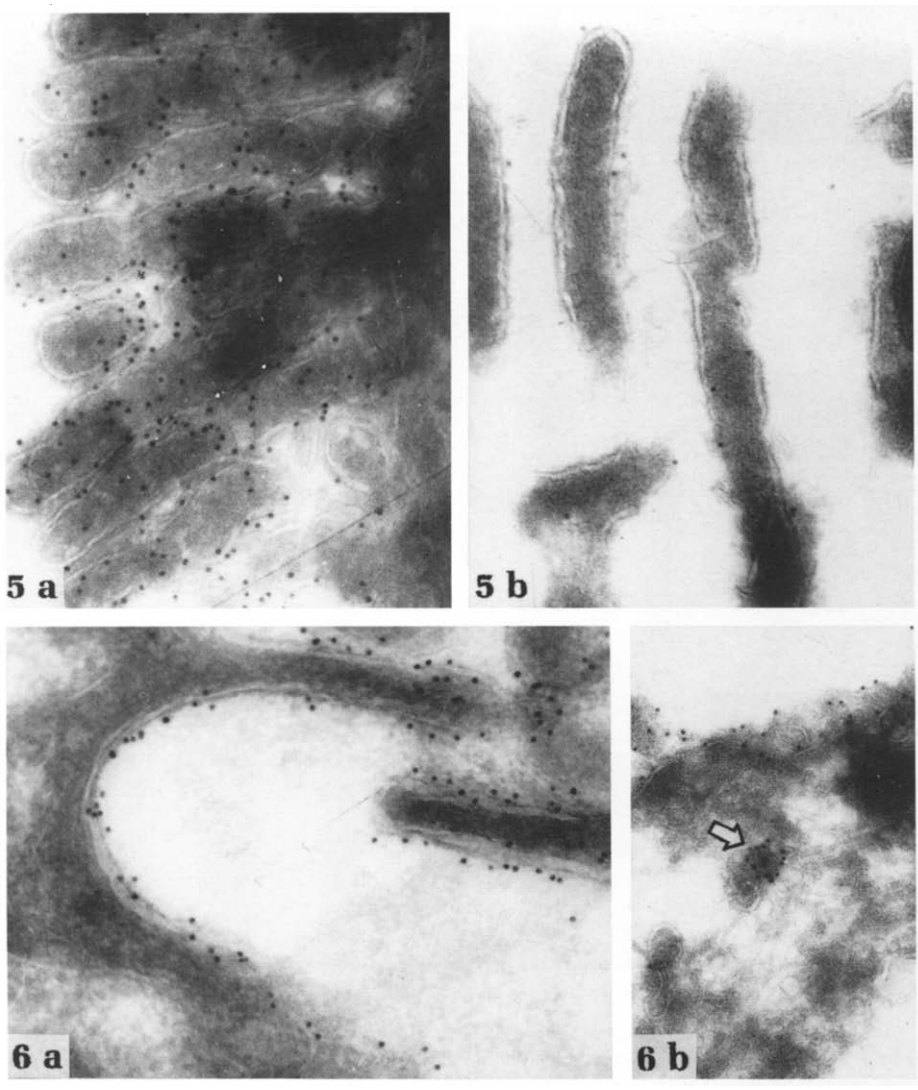

Fig. 5. Single immunolabelling of ultrathin cryosections of brush border from rat small intestinal enterocytes. a. with pRAR-APN antiserum/protein A-10 nm gold, b. with pRAR-DPP IV antiserum/protein A-10 nm gold. ×80.000. Fig. 6a, b. Single immunolabelling of ultrathin cryosections of human synovial fibroblasts with CD 13-mAB/GAM-12 nm gold. Fig. 6b shows a gold labelled intracellular vesicle (→). ×80.000.

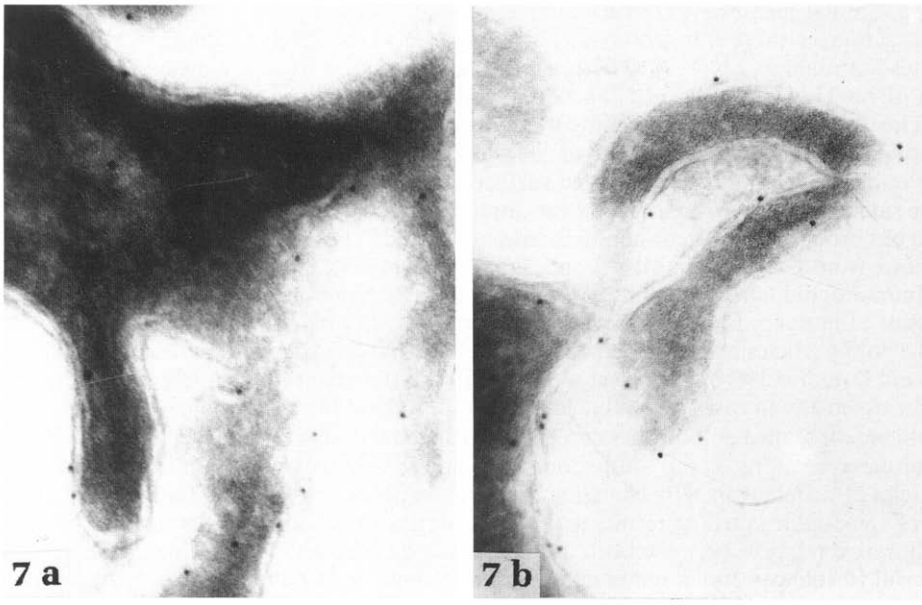

Fig. 7. Single immunolabelling of ultrathin cryosections of human T lymphocytes. a. with CD 13-mAB/GAM-12 nm gold, b. with CD 26-mAB/GAM-12 nm gold. ×80.000.

In Fig. 6 (a, b) and Fig. 7 (a, b) the detection of APN (synovial fibroblasts, T lymphocytes) and DPP IV (T lymphocytes) on cultured cells are visible. Furthermore, gold-labelled vesicle-like structures were found in the luminal part of the cytoplasm of renal proximal tubular cells (Fig. 1 b) and of synovial fibroblasts (Fig. 6 b).

Discussion

Immunogold labelling in combination with cryoultramicrotomy has been proven to be a useful method for the visualization and localization of cell surface and intracellular proteins (Mizuno, 1993). Especially the membrane preservation (Mizuno, 1993) is responsible for the precise localization of APN (CD 13) and DPP IV (CD 26) on the brush border membranes of several cells from different species (Fig. 1, 5, Kettmann et al., 1992 a, b) as well as of various cultured cells (Figs. 6, 7). Cryosections according to Tokuyasu (1973, 1989) offer a good compromise between antigenicity and ultrastructure preservation and are suitable for the detection both intracellular (Figs. 1 b, 6 b) and extracellular antigens (all figures).

Our observations (Fig. 1, 5) agree with biochemical data of Kenny and Maroux (1982), who described the abundant expression of APN and DPP VI on the brush border membrane of kidney tubular cells and small intestinal enterocytes. Bathon et al. (1992) reported the two peptidases on the cell surface of human synovial fibroblasts. Our immunological detection of APN coincides with these findings (Fig. 6) as does the localization of both ectoenzymes on activated T lymphocytes (Fig. 7 a, b) with data of Kunz et al. (1993) and Mentlein et al. (1984). But the expression of CD 13 and CD 26 on T lymphocytes is less then on synovial fibroblastes or brush border membranes.

The gold-labelled vesicle-like structures (Fig. 1 b, 6 b) may be connected with biosynthesis, co-translational glycosylation and intracellular transport of microvillar proteins

to the luminal membrane (Danielsen et al., 1987). Hansen et al. (1987) suggest that in pig enterocytes these transport vesicles operate between Golgi complex and cell membranes Kettmann et al. (1992 b) detected the same vesicular structures also in the luminal part of rat kidney tubular cells and discussed them as transport vesicles.

The two applied double labelling methods resulted in similar findings. The successive technique (see Figs. 2 a, 3 a) as well as the simultaneous procedure (see Fig. 4 a) are useful for double labelling of closely related surface antigens. Although in the two methods the same side of the grids were used for the application of both antibodies with their respective gold probe size, no cross-contamination occurred. These results correspond to informations from Geuze et al. (1981) and Tapia et al. (1983).

Immunogold labelling intensity do not necessarily represent the presence of a defined amount of antigen. Furthermore, labelling densities vary markedly according to the size of the gold particles used and densities decrease with increasing gold particle size (Giberson and Demaree 1994). This creates problems with the quantitative interpretation of the results especially in cases of double labelling. To overcoming this problem silver-enhanced ultrasmall (1 nm) gold probes can be used (Slater, 1993). Because of the sensitivity of the ultracryosections, in our study double labelling was only carried out with larger gold particles of different size. By changing the label sequence of APN and DPP IV and comparing the double labelling results with the respective single labelling we can evaluate the quantitative relation between both antigens. As has been shown for single labelling of synovial fibroblasts and T lymphocytes double labelling procedures can also be applied to gelatine embedded cells. In conclusion, using the described technique it is possible to investigate localization and colocalization of other cell surface and cytosolic antigens at the ultrastructural level.

References

Ashmun RA, and Look AT (1990) Metalloprotease activity of CD 13/aminopeptidase N on the surface of human myeloid cells. Blood **75**: 462–469

Bathon JM, Proud D, Mizutani S, and Ward PE (1992) Cultured human synovial fibroblasts rapidly metabolize kinins and neuropeptides. Clin Invest **90**: 981–991

Callebaut C, Krust B, Jacotot E, and Hovanessian AG (1993) T cell activation antigen, CD 26, as a cofactor for entry of HIV in CD^{4+} cells. Science **262**: 2045–2050

Danielsen EM, Cowell GM, Norén O, and Sjöström H (1987) Biosynthesis. In: Kenny AJ, Turner AJ (Eds) Mammalian ectoenzymes. Elsevier Science Publishers, Oxford

De la Baume S, Yi C, Schwartz J-C, Chaillet P, Marcais-Collado H, and Costentin J (1983) Participation of both "enkephalinase" and aminopeptidase activities in the metabolism of endogenous enkephalins. Neuroscience **8**: 143–151

Delmas B, Gelfi J, L'Haridon R, Vogel LJ, Sjöström H, Norén O, and Laude H (1992) Aminopeptidase N is a major receptor for the enteropathogenic coronavirus TGEV. Nature **357**: 417–419

Geuze HJ, Slot JW, Van der Ley PA, Scheffer R, and Griffith J (1981) Use of colloidal gold particles in double-labeling immunoelectron microscopy of ultrathin frozen tissue sections. J Cell Biol **89**: 653–665

Giberson RT, and Demare RS (1994) The influence of immunogold particle size on labeling density. Microscopy Res & Technique **27**: 355–357

Gossrau R (1985) Cytochemistry of membrane proteases. Histochem J **17**: 737–771

Gossrau R (1993) Proteases In: Ogawa K, Barka T (Eds) Electron microscopic cytochemistry and immunocytochemistry in biomedicine. CRC Press Inc., Boca Raton, Florida

Gros C, Giros B, and Schwartz J-C (1985) Identification of aminopeptidase M as an enkephalin-inactivation enzyme in rat cerebral membranes. Biochemistry **24**: 2179–2185

Hansen GH, Sjöström H, Norén O, and Dabelsteen E (1987) Immunomicroscopic localization of aminopeptidase N in the pig enterocyte. Implications for the route of intracellular transport. Eur J Cell Biol **43**: 253–259

Johnson RC, Zhu D, Augustin-Voss HG, and Pauli BU (1993) Lung endothelial dipeptidyl peptidase IV is an adhesion molecule for lung-metastatic rat breast and prostate carcinoma cells. J Cell Biol **121**: 1423–1432

Kenny AJ, and Maroux S (1982) Topology of microvillar membrane hydrolases of kidney and intestine. Physiol Rev **62**: 91–123

Kettmann U, Humbel B, and Holzhausen H-J (1992a) Ultrastuctural localization of dipeptidylpeptidase IV in the glomerulum of the rat kidney. Acta histochem **92**: 225–227

Kettmann U, Humbel B, Holzhausen H-J, Bahn H, and Aurich H (1992b) Immunoelectron microscopic localization of microsomal alanine aminopeptidase. Acta histochem **93**: 333–340

Kunz D, Bühling F, Hütter H-J, Aoyagi T, and Ansorge S (1993) Aminopeptidase N (CD 13, EC 3.4.11.2.) occurs on the surface of resting and concanavalin A-stimulated lymphocytes. Biol Chem Hoppe-Seyler **374**: 291–296

Laurell CB (1965) Antigen-antibody crossed electrophoresis. Anal Biochem **10**: 358–361

Look AT, Ashmun RA, Shapiro LH, and Peiper Sc (1989) Human myeloid plasma membrane glycoprotein CD 13 (gp 150) is identical to aminopeptidase N. J Clin Invest **83**: 1299–1307

McDonald JK, and Barrett AJ (1986) Mammalian proteases. A glossary and bibliography. Vol. 2: Exopeptidases. Academic Press, London

Mentlein R, Heymann E, Scholz W, Feller AC, and Flad H-D (1984) Dipeptidyl peptidase IV as a new surface marker for a subpopulation of human T-lymphocytes. Cell Immunol **89**: 11–19

Mizuno M (1993) Electron microscopic immunocytochemistry. Preservation of immunoreactivity and ultrastructure. In: Ogawa K, and Barka T (Eds) Electron microscopic cytochemistry and immunocytochemistry in biomedicine. CRC Press Inc., Boca Raton, Florida

Nausch I, Mentlein R, and Heymann E (1990) The degradation of bioactive peptides and proteins by dipeptidyl peptidase IV from human placenta. Biol Chem Hoppe-Seyler **371**: 1113–1118

Riemann D, Schwachula A, Hentschel M, and Langner J (1993) Demonstration of CD 13/Aminopeptidase N on synovial fluid T cells from patients with different forms of joint effusions. Immunbiol **187**: 24–35

Saiki I, Fujii H, Yoneda J, Abe F, Nakajima M, Tsuruo T, and Azuma I (1993) Role of aminopeptidase N (CD 13) in tumor-cell invasion and extracellular matrix degradation. Int J Cencer **54**: 137–143

Slater M (1993) Ultrastructural double labelling using colloidal gold. Micron **24**: 661–675

Stein H, Schwarting R, and Niedobitek G (1989) Cluster report: CD 26. In: Knapp W (Ed) Leucocyte typing IV. University Press, Oxford

Tapia FJ, Varndell IM, Probert L, De Mey J, and Polak JM (1983) Double immunogold staining method for the simultaneous ultrastructural localization of regulatory peptides. J Histochem Cytochem **31**: 977–981

Tokuyasu KT (1989) Use of poly(vinylpyrrolidone) and poly(vinylalcohol) for cryoultramicrotomy. Histochem J **21**: 163–171

Tokuyasu KT (1993) A technique for ultracryotomy of cell suspensions and tissues. J Cell Biol **57**: 557–565

Ulmer AJ, Mattern T, Feller AC, Heymann E, and Flad HD (1990) CD 26 antigen is a surface dipeptidyl peptidase IV (DPP IV) as characterized by monoclonal antibodies clone TII-19-4-7 and 4EL1C7. Scand J Immunol **31**: 429–435