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Neutral endopeptidase-24.11 (enkephalinase)

Biosynthesis and localization in human fibroblasts

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The biosynthesis, glycosylation and subcellular localization of the neutral endopeptidase-24.11 were studied in cultured human fibroblasts. The enzyme was synthesized as a precursor (M_r 88000) containing four or five *N*-linked oligosaccharides. Within 1 h the synthesis-mature (M_r 94000) endopeptidase-24.11 was formed and contained sialylated oligosaccharides. The half-life of endopeptidase-24.11 was 3.7 days and in the presence of 10 mM-NH_4Cl it increased to 6 days. Mature endopeptidase-24.11 was solubilized with 0.2% saponin and partitioned into Triton X-114. In intact fibroblasts, endopeptidase-24.11 was accessible to antibodies and to neuraminidase even when the treatment was performed at 4 °C. The localization of endopeptidase-24.11 to the plasma membrane in cultured fibroblasts was further demonstrated by immunocytochemistry.

INTRODUCTION

Endopeptidase-24.11 or as frequently referred to in literature 'enkephalinase' (EC 3.4.24.11) is a neutral metallopeptidase (Kerr & Kenny, 1974) typically localized in microvillar membranes of various epithelia (Kerr & Kenny, 1974; Danielsen *et al.*, 1980; Johnson *et al.*, 1985*a*; Erdös *et al.*, 1985; Bowes & Kenny, 1986). Endopeptidase-24.11 was also found in skin and lung fibroblasts (Johnson *et al.*, 1985*a*), brain cells (Almenoff *et al.*, 1981; Schwartz *et al.*, 1981; Horsthemke *et al.*, 1983) and human neutrophils (Connelly *et al.*, 1985). However, it is not a ubiquitous enzyme (Gee *et al.*, 1985); for example, its activity in endothelial cells or blood plasma is very low (Johnson *et al.*, 1985*a*,*b*; Almenoff *et al.*, 1984). Endopeptidase-24.11 cleaves a variety of biologically active peptides including enkephalins (Hersh, 1982; Schwartz *et al.*, 1981), bradykinin, neurotensin and substance P (Almenoff *et al.*, 1981; Gafford *et al.*, 1983; Skidgel *et al.*, 1984; Matsas *et al.*, 1984; Stephenson & Kenny, 1987), fMet-Leu-Phe (Connelly *et al.*, 1985) and other active peptides. The biosynthesis of endopeptidase-24.11 has been studied in pig kidney cortex slices (Stewart & Kenny, 1984). It is synthesized as a glycosylated precursor that is sensitive to endoglucosaminidase H. The precursor is processed to a larger-molecular-mass mature form containing complex oligosaccharides and is transported to the apical membrane.

Measurement of endopeptidase-24.11 levels in blood may also be of clinical interest as its activity is increased about 3-fold in sarcoidosis (Almenoff *et al.*, 1984), and up to 60-fold in the sera samples of patients with adult respiratory distress syndrome (Johnson *et al.*, 1985*b*). Here we report on the biosynthesis of human endopeptidase-24.11 in cutured cells and on the immunocytochemistry of the enzyme.

EXPERIMENTAL

Cell culture

Human diploid skin fibroblasts and human HepG2 hepatoma cells were maintained at 37 °C in a mixture of air and CO_2 (19:1) in Eagle's minimum essential medium supplemented with antibiotics, non-essential amino acids and 7.5% (v/v) fetal calf serum (Boehringer Mannheim) as described by Cantz *et al.* (1972). Human monocytes U937 (Sundström & Nilsson, 1967) and promyelocytes HL-60 (Collins *et al.*, 1977) were cultured in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum.

Labelling of cells

Fibroblasts and HepG2 cells grown in 34 mm-diameter Petri dishes were labelled in a modified Waymouth medium (von Figura *et al.*, 1983) and U937 and HL-60 cells in methionine-free RPMI 1640 medium (Gupta *et al.*, 1984). For labelling L-[^{35}S]methionine, with a specific activity of 44 TBq/mmol (from Amersham-Buchler, Braunschweig, Germany), and L-[^{3}H]mannose, with a specific activity of 810 GBq/mmol (DuPont, Dreieich, Germany) were used. The labelling with mannose was performed in glucose-free medium (Hasilik & von Figura, 1981).

Treatment of cells with enzymes

Trypsin: the cells were washed twice with Hanks balanced salt solution and incubated with 0.1% trypsin in 0.14 M-NaCl/10 mM-Na phosphate, pH 7.4 (phosphate-buffered saline), as indicated. The cells were collected by centrifugation and washed with phosphate-buffered saline supplemented with 1.5% and then with 0.5% fetal calf serum. Neuraminidase: the cells were

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washed twice with 2 mM-$CaCl_2$, 140 mM-NaCl, 5 mM-glucose, 1 mg of bovine serum albumin/ml and 20 mM-Na phosphate, pH 6.2. The incubation was performed with 3 munits of *Vibrio cholerae* neuraminidase in 400 μl of this buffer as indicated and terminated by a wash with cold phosphate-buffered saline.

Cross-linking

Labelled cells were washed with Hanks balanced salt solution and incubated with 1 ml of Hanks solution containing 2% dimethyl sulphoxide either alone or with 1 mM-dithiobis-(succinimidyl propionate) for 10 min at 37 °C (from Pierce, Weiskirchen, Germany). The mixture containing the reagent was neutralized with 5 μl of 0.1 M-NaOH after 2–3 min of incubation.

Immunoprecipitation

After labelling, the cells were lysed in 1 ml of buffer A containing 1% Triton X-100, 0.5% Na deoxycholate, 0.2% SDS, 5 mg of bovine serum albumin/ml, 1 mM-phenylmethanesulphonyl fluoride, 5 mM-iodoacetamide, 1 mM-$MgCl_2$, 20 μg of DNAase, 0.14 M-NaCl and 10 mM-Na phosphate, pH 7.4. The medium was mixed 1:1 with a 2-fold concentrated buffer A. After incubating for 15 min, EDTA was added to a final concentration of 2 mM and the samples were pre-adsorbed with 50 μl of a 10% *Staphylococcus aureus* cell-wall preparation (Immuno Precipitin, Gibco BRL, Eggenstein, Germany) for 30 min on ice. The pre-adsorption was repeated and the supernatants were frozen and thawed and then centrifuged at 45000 ***g*** for 1 h. The supernatants were mixed with 2 μl of rabbit antiserum to endopeptidase-24.11 (Erdös *et al.*, 1985; Johnson *et al.*, 1985*a*) or anti β-hexosaminidase B (von Figura & Weber, 1978) antiserum, or with affinity-purified rabbit antibodies to cathepsin D (Gieselmann *et al.*, 1983). The immune complexes were collected after an overnight incubation at 4 °C. Washed immunoprecipitin (von Figura *et al.*, 1985), 0.2 mg/μg of antibody, was added and the samples were rotated for 1 h. The solids were collected by centrifugation and washed with 800 μl of (1) buffer A without the inhibitors, $MgCl_2$ and the enzyme, (2) the same mixture but substituting 2 M-KCl for SDS, (3) 0.6 M-NaCl, 0.1% SDS, 0.05% Nonidet NP40 and 10 mM-Tris/HCl at pH 8.6. This was followed by washing twice with 14 mM-NaCl/1 mM-Na phosphate, pH 7.4. The precipitate was heated with 65 μl of 1% of SDS, 10 mM-dithiothreitol and 0.125 M-Tris/HCl (pH 6.8, 95 °C) for 6 min and the extract was cleared by centrifugation.

The separation of cellular endopeptidase-24.11 accessible to antibodies was done by incubating fibroblasts in 1 ml of standard culture medium with 5 μl of anti-endopeptidase-24.11 antiserum as indicated. After incubation the cells were washed twice with phosphate-buffered saline. Cells with the adsorbed antibody were lysed in 1 ml of buffer A containing the homogenate from one dish. The homogenate was then subjected to preabsorption with *Staphylococcus aureus* cell walls as described above; in this step the endopeptidase-24.11 molecules that were complexed with the antibody were removed.

Digestions *in vitro*

To treat the immunoprecipitated endopeptidase-24.11 with glycosidases, the antigen was solubilized from the washed cell walls by heating it in 20 μl of 0.5% SDS, 2.5 mM-dithiothreitol and 25 mM-Na phosphate for 10 min at pH 5.4. Dithiothreitol was omitted from neuraminidase experiments. The samples were diluted with 85 μl of water and supplemented with 2.5 μl of 1 M-Na phosphate, pH 5.3 (for endoglucosaminidase H), or 20 μl of 5 mM-$CaCl_2$ containing 5 mg of bovine serum albumin/ml (for neuraminidase). The samples were cleared up by centrifugation and the supernatants divided for control and glycosidase treatment. Endoglucosaminidase H was used for 20 h at 37 °C with 1 munit of enzyme from Health Center (Albany, New York), unless otherwise stated, and the neuraminidase treatment was incubated for 90 min at 37 °C with 1.25 munit of enzyme from *Vibrio cholerae* (Calbiochemicals GmbH, Frankfurt). To the treated samples, 25 μl of 28 mM-dithiothreitol in 2.8% SDS, 28.6% glycerol and 0.35 M-Tris/HCl, pH 6.8, was added and then they were heated at 95 °C for 5 min.

Separation of the labelled antigens

The solubilized immunoprecipitates were electrophoresed in 12.5% polyacrylamide gels according to Laemmli (1970) and Hasilik & Neufeld (1980). The gels were developed by fluorography (Laskey & Mills, 1975). For quantitative determination the bands were excised and the radioactivity counted (Waheed *et al.*, 1982).

Immunocytochemistry

For immunogold localization, fibroblasts were scraped off the dish after fixation with 2% paraformaldehyde in 50 mM-Na phosphate, pH 7.4. Ultra-thin cryosections were prepared and indirectly labelled (Geuze *et al.*, 1985) using rabbit anti-endopeptidase-24.11 antiserum, swine anti-rabbit IgG antiserum and 9 nm gold particles successively. Sections were stained with uranyl acetate and embedded in methyl cellulose as described by Slot & Geuze (1984).

RESULTS

Biosynthesis, maturation and stability

The expression, synthesis, glycosylation and stability of endopeptidase-24.11 was examined in several human cell lines by immunoprecipitation from extracts of metabolically labelled cells. In contrast to fibroblasts, in hepatoma HepG2 cells, monocytes U937 and promyelocytes HL-60, no radioactive endopeptidase-24.11 was found although all these cell lines contained comparable amounts of cathepsin D (Fig. 1*a*). Labelling of endopeptidase-24.11 with [2-^{3}H]-mannose indicated that it is a glycoprotein although its carbohydrate content was resistant to cleavage by endoglucosaminidase H. In contrast, cathepsin D present in the same cell sample was sensitive to endoglucosaminidase H (Fig. 1*b*).

Shortly after the synthesis endopeptidase-24.11 was sensitive to endoglucosaminidase H (Fig. 2). This was observed in an experiment in which the cells were labelled for 15 min and endopeptidase-24.11 was isolated after chase periods of various lengths. Initially the enzyme had an M_r of 88000. After treatment with endoglucosaminidase H the M_r decreased to 78000. But approx. 1 h after the synthesis the M_r of endopeptidase-24.11 increased from 88000 to 94000 and this increase was correlated with a loss of sensitivity to endoglucos-

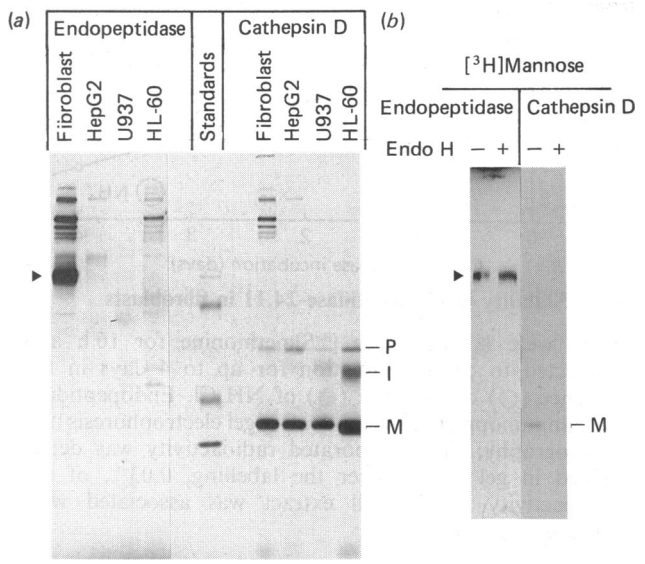

Fig. 1. Synthesis and glycosylation of endopeptidase-24.11 in cultured human cells

(*a*) Fibroblasts, HepG2 cells, U937 monocytes and HL-60 promyelocytes were labelled with 600 kBq of [^{35}S]-methionine for 20 h. In the extracts of cells 200, 220, 220 and 160 kBq were recovered. From portions split into 3/4 and 1/4, endopeptidase-24.11 and cathepsin D were precipitated respectively. (*b*) Fibroblasts were labelled with 1.5 MBq of [2-^{3}H]mannose for 19 h. 0.16 MBq was recovered in the cell extract. Endopeptidase-24.11 and cathepsin D were precipitated sequentially. After solubilization portions were incubated with and without endoglucosaminidase H (EndoH) as indicated. The labelled proteins were separated by polyacrylamide electrophoresis and visualized by fluorography. The position of endopeptidase-24.11 is shown by an arrowhead. Precursor (P), intermediate (I) and mature (M) forms of cathepsin D are marked. The bands of M_r 150000 or more occur in both immunoprecipitates and are considered a contamination. The marker proteins, from the top, were phosphorylase B, bovine serum albumin, ovalbumin and carbonic anhydrase with M_r values of 97400, 69000, 46000 and 30000 respectively.

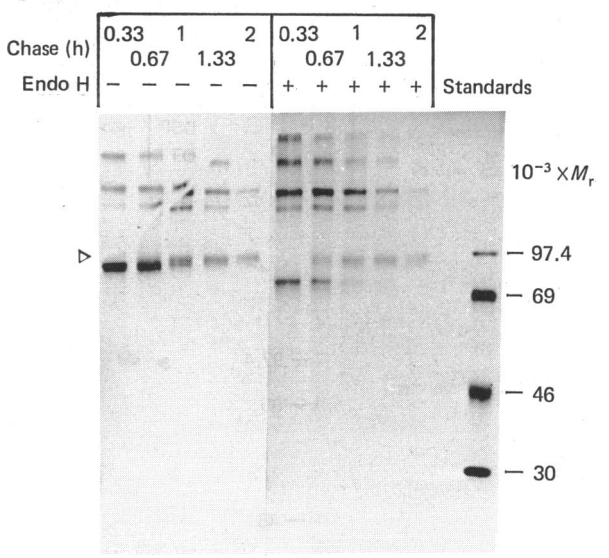

Fig. 2. Maturation of endopeptidase-24.11

Medium with [^{35}S]methionine, 1.85 MBq, was used to label fibroblasts sequentially for 15 min each. The cultures were harvested after 20–120 min chase. Endopeptidase-24.11 was precipitated and portions were incubated without or with endoglucosaminidase H.

aminidase H, suggesting a processing of the high mannose to a complex-type oligosaccharide. The cleavage of the oligosaccharides from endopeptidase-24.11 occurred in several rather distinct steps (Fig. 3*a*). From the changes in the M_r due to deglycosylation it was concluded that endopeptidase-24.11 contains four or five *N*-linked oligosaccharides.

Endopeptidase-24.11 is likely to contain intra-chain disulphide bridges as indicated by an enhancement of the apparent M_r due to reduction (Fig. 3*b*). When cross-linked with dithiobis-(succinimidyl propionate) (Fig. 3*b*), endopeptidase-24.11 formed large products with a prominent species with an M_r of approx. 200000, which may correspond to dimers or trimers.

To determine the stability of mature endopeptidase-24.11 the enzyme was precipitated from extracts of labelled cells that were subjected to chase incubations of up to 4 days. From a semi-logarithmic plot, a half-life of 3.7 days was estimated (Fig. 4). In the presence of 10 mM-NH_4Cl the half-life was prolonged to 6 days.

Association with membranes

Most of the endopeptidase-24.11 sedimented in homogenates prepared by sonication of fibroblasts in a low-ionic-strength buffer. The enzyme was rendered soluble with Triton X-100 and also with the mild steroid detergent saponin. Extensive though not complete solubilization was achieved at 0.05% saponin (not shown).

In partition experiments (performed essentially as described by Conzelmann *et al.*, 1986) the labelled enzyme fractionated with Triton X-114 (not shown). When fibroblasts were cultured in the absence of serum with radioactive palmitic acid, a weak labelling of endopeptidase-24.11 was observed. The label corresponded to $\leqslant$ 10 p.p.m. of cell-associated radioactivity and the structure of the labelled moiety could not be analysed.

Subcellular localization

Endopeptidase-24.11 was not appreciably affected by treating fibroblasts with trypsin (Fig. 5*a*). Resistance to trypsin has been previously observed with the human (Gafford *et al.*, 1983) and pig kidney (Kenny *et al.*, 1983) enzymes. Neuraminidase, however, diminished the apparent molecular mass of the endopeptidase-24.11 in fibroblasts and this change was observed both at 37 °C and 4 °C, i.e. in the absence of exchange between cell surface and intracellular membranes (Fig. 5*b*). Thus, a majority of endopeptidase-24.11 was exposed on the plasma membrane of the cells. This was documented further by showing that the antigen in intact cells was accessible to the antibody at both 37 °C and 4 °C (Fig. 5*c*). After binding of the antibody, the complexes were removed from the cell extracts by incubation with *Staphylococcus aureus* cell walls. Finally, our immunocytochemical data (Fig. 6) confirm that endopeptidase-24.11 is present predominantly at the plasma membrane

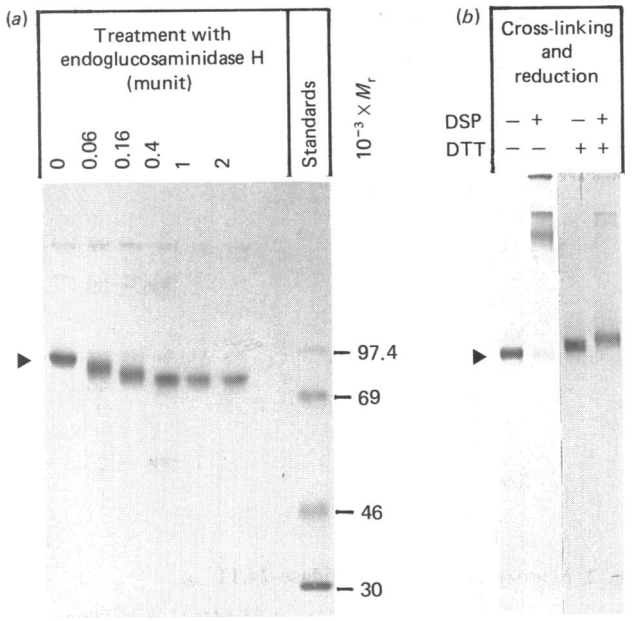

Fig. 3. Deglycosylation of the precursor and cross-linking of the mature endopeptidase-24.11

(*a*) Fibroblasts in two dishes were labelled with 1.85 kBq of [^{35}S]methionine for 10 min and endopeptidase-24.11 was precipitated. Portions of a combined extract were incubated with up to 2 munits of endoglucosaminidase H for 20 h at 37 °C. The position of the undigested endopeptidase-24.11 is indicated by an arrowhead. (*b*) Fibroblasts in 2 dishes were labelled with 400 kBq of [^{35}S]methionine for 22 h. One dish was treated with dithiobis-(succinimidyl propionate) (DSP). Endopeptidase-24.11 was precipitated and the labelled enzyme was solubilized in the absence of dithiothreitol. Portions were supplemented with 10 mM-dithiothreitol (DTT) and incubated for 6 min at 95 °C. The position of the mature non-cross-linked non-reduced endopeptidase-24.11 is marked by an arrowhead.

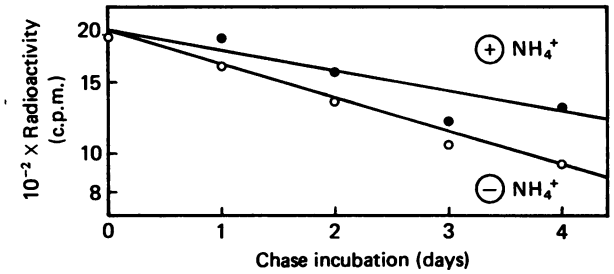

Fig. 4. Stability of endopeptidase-24.11 in fibroblasts

Cells were labelled with [^{35}S]methionine for 16 h and subjected to chase incubation for up to 4 days in the absence (○) or presence (●) of NH_4Cl. Endopeptidase was immunoprecipitated and, after gel electrophoresis and fluorography, the incorporated radioactivity was determined in gel slices. After the labelling, 0.03% of the radioactivity in the cell extract was associated with endopeptidase-24.11.

in fibroblasts. A minor fraction of the enzyme may be associated with intracellular vacuolar profiles.

DISCUSSION

This study demonstrates that human cultured fibroblasts are a useful model in studies on endopeptidase-24.11 although, at least in pig tissues, fibroblastic cells appear to express this enzyme relatively poorly (Bowes & Kenny, 1987). Among several human cell types examined in this study, only fibroblasts synthesized endopeptidase-24.11, while all cells synthesized and processed cathepsin D in a similar way to fibroblasts (Hasilik & Neufeld, 1980). Human endopeptidase-24.11 is synthesized as an M_r 88000 protein containing four or five *N*-linked oligosaccharides. About 1 h after the synthesis these oligosaccharides become resistant to endoglucosaminidase H. This change is accompanied by an increase in the M_r of the enzyme to 94000 and indicates formation of complex oligosaccharides. The presence of sialic acid, a component of the compex oligosaccharides in the mature enzyme, can indirectly be demonstrated by a reduction in M_r due to digestion with neuraminidase. Previously, it has been observed that human prostate endopeptidase-24.11 is sensitive to sialidase, while the enzyme from human kidney is not (Erdös *et al.*, 1985). The variability in the carbohydrate content in human endopeptidase-24.11 resembles that in the enzyme purified from different pig tissues (Fulcher *et al.*, 1983; Relton *et al.*, 1983).

The synthesis and maturation of human endopeptidase-24.11 appears to be similar to that of the pig enzyme, which has been studied by Stewart & Kenny (1984). In pig kidney slices endopeptidase-24.11 is synthesized as an M_r 88000 endoglucosaminidase-sensitive glycoprotein that matures to an M_r 93000 resistant form. Our data on human endopeptidase-24.11 suggest that the post-translational modification of this enzyme involves formation of intra-chain disulphide bridges, since reduction of the denatured enzyme results in a decrease in the electrophoretic mobility (Fig. 3).

When the cells are treated with dithiobis-(succinimidyl propionate) a large proportion of the mature enzyme becomes cross-linked leading to products with M_r values of 200000 or more. This indicates that in fibroblasts, endopeptidase-24.11 forms dimers or larger oligomers. Based on gel filtration, Fulcher & Kenny (1983) proposed that the enzyme from pig kidney is a dimer.

In cultured human fibroblasts endopeptidase-24.11 has a half-life of 3.7 days. In the presence of 10 mM-NH_4Cl the half-life is prolonged to 6 days. NH_4Cl is a lysosomotropic amine, which perturbs the acidification and the digestion in lysosomes (Mayer & Doherty, 1986). However, NH_4Cl also inhibits endocytosis (Sando *et al.*, 1979), secretion (Neblock & Berg, 1982) and probably membrane transport in general (Matlin, 1986; Dean *et al.*, 1984). It is likely that the stabilization of endopeptidase-24.11 is due to inhibition of membrane transport into the lysosomes.

Both in human (this study) and in pig (Matsas *et al.*, 1985) cells endopeptidase-24.11 is an integral membrane protein as indicated by partition into Triton X-114. In pig kidney endopeptidase-24.11, a hydrophobic sequence localized in the vicinity of the *N*-terminus has been suggested to anchor the enzyme in the membrane (Fulcher *et al.*, 1986).

In pig kidney and intestine (Gee *et al.*, 1983) as well as in human kidney and prostate (Erdös *et al.*, 1985) endopeptidase-24.11 has been demonstrated on the

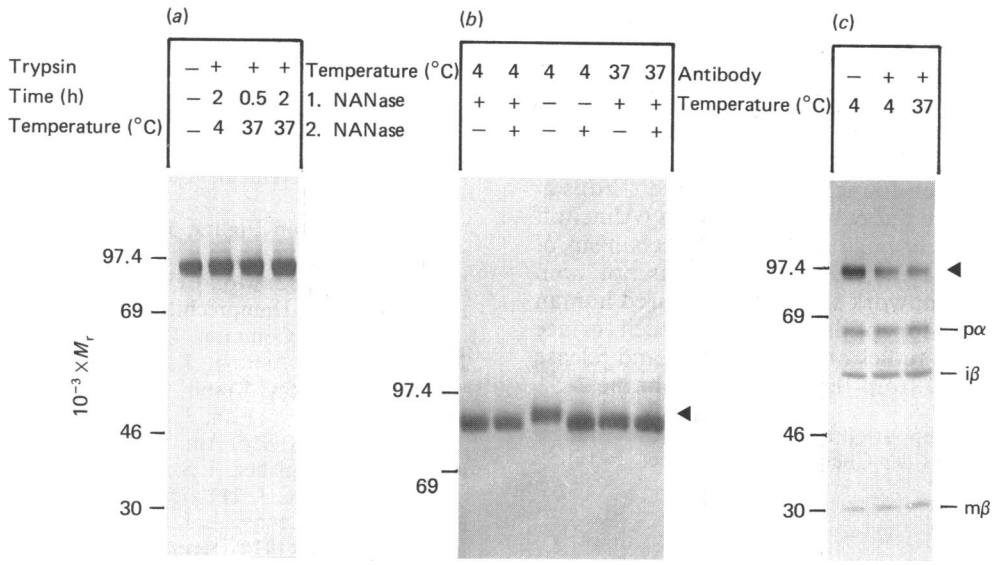

Fig. 5. Accessibility of endopeptidase-24.11 to medium

In three experiments fibroblasts were labelled with 370-480 kBq of [^{35}S]methionine for 17–22 h. The cells were treated with trypsin, neuraminidase (NANase) or anti-endopeptidase-24.11 antibody. (*a*) Endopeptidase-24.11 was isolated from cells that were treated or untreated for 0.5 h or 2 h with trypsin at 4 °C or 37 °C as indicated. (*b*) Endopeptidase-24.11 was isolated from cells that were untreated or treated for 2 h with neuraminidase (1. NANase) at 4 °C or 37°, and portions were incubated with neuraminidase (2. NANase). (*c*) Fibroblasts were incubated with anti-endopeptidase-24.11 antiserum for 2 h. After separation of the immunocomplexes endopeptidase-24.11 and β-hexosaminidase were immunoprecipitated concomitantly. The positions in the gels of endopeptidase-24.11 (arrowhead) and of α-chain precursor (pα), β-chain intermediate (iβ) and mature β-chain (mβ) of β-hexosaminidase are indicated.

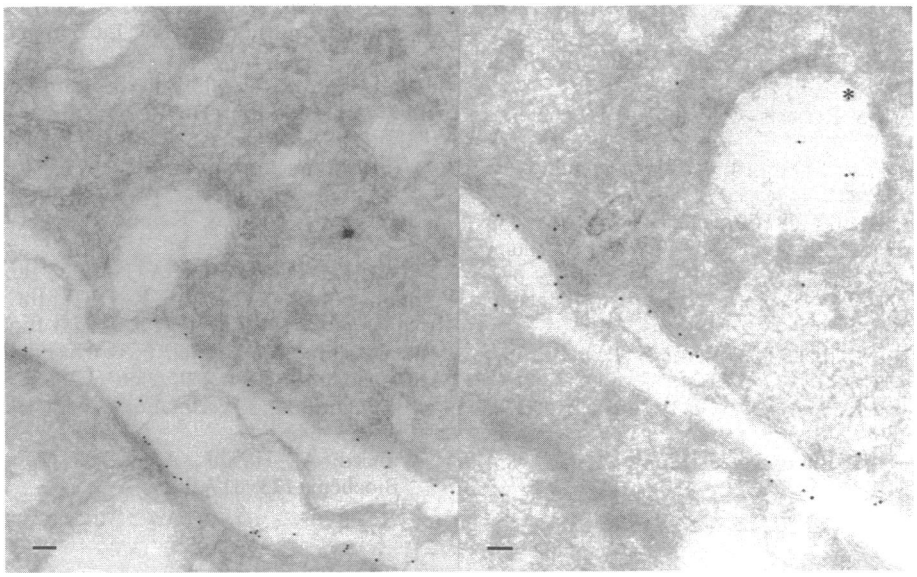

Fig. 6. Immunocytochemical localization of endopeptidase-24.11

Immunogold localization of endopeptidase-24.11 is shown in two cryo-sections of paraformaldehyde-fixed fibroblasts. The gold label was found mainly at the plasma membrane. The profile marked with an asterisk may represent an invagination of the plasma membrane or a lysosomal compartment. The length of the bar represents 100 nm.

luminal side of the epithelia. In human endothelial cells and in foreskin fibroblasts immunohistochemistry showed a punctate distribution along the filopodia and in the cytoplasm (Johnson *et al.*, 1985*a*). Bowes & Kenny (1986) have also observed a predominantly granular intracellular localization of the enzyme in fibroblastic cells in early cultures from pig lymph nodes. The present study indicates that in cultured human fibroblasts most of the enzyme is exposed at the cell surface. Stimulated by this difference we have examined the distribution of

endopeptidase-24.11 in skin fibroblasts with immunocytochemistry and observed predominantly cell surface localization of the enzyme. It appears that the localization of endopeptidasde-24.11 in fibroblast depends on the origin of the cells and the conditions of culture.

Endopeptidase-24.11 has been detected in certain body fluids. It has been found in seminal fluid (Erdös *et al.*, 1985) and in some patients also in serum (Almenoff *et al.*, 1984; Johnson *et al.*, 1985 *b*). The mechanism of the release of the enzyme from the cells has not been elucidated. The present work shows that cultured human fibroblasts contain endopeptidase-24.11 at the cell surface and may be useful in studies on the transport and release of this protein to and from the plasma membrane.

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