Identification of cell surface dipeptidylpeptidase IV in human fibroblasts

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An antigen with dipeptidylpeptidase IV activity was identified at the surface of normal human fibroblasts. Hydrophobic interaction electrophoresis in phenyl-Sepharose revealed that the enzyme contained a hydrophobic domain, while lactoperoxidase-catalysed iodination with $^{125}$I of living cells indicated that the protein was located at the cell surface. Crossed immunoelectrophoresis with specific antibodies of acid-extracted or papain-treated cells showed a shift of the dipeptidylpeptidase IV peak to a faster mobility. The molecular properties of the fibroblast enzyme were clearly different from those described for dipeptidylpeptidase IV from other tissues and species. Fibroblast dipeptidylpeptidase IV contained two different disulphide-linked subunits, of apparent $M_r$ values 125 000 and 135 000 (denatured and reduced). In gel filtration, an $M_r$ of about 400 000 was observed for the unreduced molecule. The enzymic properties of fibroblast dipeptidylpeptidase IV were very similar to those of the well-characterized pig kidney enzyme. Activity towards glycyl-L-prolyl-$\beta$-naphthylamide was inhibited 50% by 0.023 mM-di-isopropylphosphorofluoridate. L-Alanyl-L-alanyl-$\beta$-naphthylamide was hydrolysed ten times more slowly than glycyl-L-prolyl-$\beta$-naphthylamide.

Recently, Verlinden et al. (1981a) described the application of crossed immunoelectrophoresis to the characterization of cellular antigens of cultured fibroblasts. Membrane-bound aminopeptidase M was identified in the crossed immunoelectrophoresis pattern of normal human fibroblasts, with a polypeptide anti-(human fibroblast) serum (Verlinden et al., 1981b,c). Characteristically, this antigen migrated faster in crossed immunoelectrophoresis when cells had been extracted at pH 5.5. When the effect of acid treatment was further examined, another antigen which behaved similarly to aminopeptidase was detected.

Here we present the characteristics of this antigen. The immunoprecipitate stained enzymically in the presence of Gly-L-Pro-$\beta$-Nap, a substrate specific for dipeptidylpeptidase IV (EC 3.4.14.5) (Hopsu-Havu & Sarimo, 1967). When the structural and enzymic properties of this fibroblast dipeptidylpeptidase IV were examined and compared with those described for enzymes from other tissues, important differences were noted in molecular weight and subunit composition.

Experimental

Materials

Phenyl-Sepharose CL-4B was purchased from Pharmacia. Berol EMU-043 was from Berol Kemi AB, Sweden. Gly-L-Pro-$\beta$-Nap, L-Ala-L-Ala-$\beta$-Nap and L-Ala-$\beta$-Nap came from Bachem Feinchemikalien AG, Bubendorf, Switzerland. Papain was from Sigma. Pansorbin beads were purchased from Calbiochem-Behring, and di-isopropylphosphorofluoridate from Aldrich. Peroxidase-linked swine anti-(rabbit IgG) sera were from Dakopatts, Copenhagen, Denmark. [35S]Methionine (1240 Ci/mmol), [14C]Fucose (250 mCi/mmol) and Na$^{125}$I (100 mCi/ml) were from The Radiochemical Centre, Amersham.

Bestatin was a gift from Dr. H. Umezawa, Institute of Microbial Chemistry, Microbial Chemistry Research Foundations, Tokyo, Japan.

Crossed immunoelectrophoresis

Crossed immunoelectrophoresis was done as described (Verlinden et al., 1981a). Hydrophobic-interaction crossed immunoelectrophoresis was performed according to the procedure of Bjerrum (1978), in which phenyl-Sepharose CL-4B beads were used.
were incorporated in the first-dimension agarose gel. Berol (1%) was present in the second-dimension gel.

The immunoprecipitates were stained with Coomassie Brilliant Blue R-250 as described by Verlinden et al. (1981a). Enzymic staining of immunoprecipitates for dipeptidylpeptidase IV (or aminopeptidase M) activity was performed on wet gels by coupling β-naphthylamide, the product from splitting Gly-Pro-β-Nap (or Ala-β-Nap), to Fast Blue B salt according to Nachlas et al. (1957). Enzymic staining for maltase, isomaltase and sucrase, performed on crossed immunoelectrophoresis plates, was as described by Danielsen et al. (1977). The procedure for neutral endopeptidase activity staining on crossed immunoelectrophoresis plates was from Booth et al. (1979). Autoradiography was done as described by Verlinden et al. (1981a).

Preparation of cells for crossed immunoelectrophoresis

Cell culture and harvesting of human diploid fibroblasts (NHF) by trypsinization was done as described by Verlinden et al. (1981a). The resulting cell pellets were stored at −20°C. Extracts were prepared by one of the following procedures.

(a) Detergent extraction. The frozen cell pellets were thawed, resuspended in 4 vol of 75 mM-Veronal buffer (pH 8.6) containing 2% Berol (room temperature) and sonicated immediately. Insoluble material was removed by centrifugation in a Beckman Airfuge at 130000 g (10^4 rev./min) for 6 min.

(b) Acid-detergent extraction. Thawed cell pellets were resuspended in 4 vol of 0.16 M-sodium acetate buffer (pH 5.5) containing 2% Berol. The suspension was sonicated and incubated for 60 min at 37°C. Insoluble material was removed by centrifugation as described above.

(c) Papain extraction. Papain (0.01% and 0.1%) was activated in phosphate-buffered saline (137 mM-NaCl, 8.1 mM-Na2HPO4, 1.5 mM-KH2PO4, 2.7 mM-KCl, 15 mM-NaCl), containing 0.02% EDTA and 5 mM-cysteine, by incubation at 37°C for 30 min. The thawed cell pellets were resuspended in 4 vol. of phosphate-buffered saline and sonicated. This preparation was centrifuged (130000 g for 6 min), and the pellet washed once with phosphate-buffered saline. This pellet was resuspended in the activated papain solution, in the presence or absence of 2% Berol as specified, and incubated for 30 min at 37°C. After addition of 270 mM-iodoaceticamide, insoluble material was removed by centrifugation as described above.

Radioactive labelling of cells

Cellular antigens were labelled either metabolically by incorporation of [35S]methionine and [14C]-fucose, or exogenously by enzymic iodination. [35S]Methionine was added at 8 μCi/ml to methionine-free Dulbecco’s modified Eagle’s medium (Flow Laboratories) containing 10% (v/v) newborn calf serum. After 24 h incubation in this medium, the cells were washed in phosphate-buffered saline, collected and treated as above.

Metabolic carbohydrate labelling was carried out by incubation of the cell layers with serum-free medium supplemented with [14C]fucose (1.2 μCi/ml) for 6 h.

Lactoperoxidase-catalysed iodination with Na125I was carried out as described (Hynes, 1973).

Preparation of antisera

Anti-fibroblast serum (aNHFiv) was obtained by intravenous injection of rabbits with whole cells as described (Verlinden et al., 1981b).

Antibodies directed against dipeptidylpeptidase IV were obtained as follows. Immunoprecipitates of the acid-extracted form of dipeptidylpeptidase IV were excised from an unstained crossed immunoelectrophoresis agarose gel under dark-field illumination and suspended in 3 vol. of water by sonication. Immunoprecipitates, equivalent to 5 × 10^6 cells, were mixed with 1 ml of complete Freund’s adjuvant and injected intradermally into rabbits at 2 week intervals. After three injections the rabbits were bled. Hyperimmune IgG was isolated as described by Verlinden et al. (1981a).

Polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed as described by Laemmli (1970) on gradient slab gels (6–20% and 4–15% polyacrylamide). Immunoprecipitates, excised from a wet crossed immunoelectrophoresis gel as described above, were dissolved in sample buffer containing 2% SDS and 5% 2-mercaptoethanol by boiling for 5 min.

Protein bands were electrophoretically transferred from polyacrylamide gels to nitrocellulose filters (Millipore) as described (Towbin et al., 1979). The filters were inactivated overnight in phosphate-buffered saline containing 0.5% casein at 37°C. Incubation with specific antibodies (diluted 1:20 in phosphate-buffered saline/casein) was for 1 h at room temperature. The filter paper was subsequently washed three times in phosphate-buffered saline/casein (10 min each) and treated with peroxidase-linked swine anti-(rabbit IgG) (1:50 dilution). After 1 h at room temperature the filters were washed three times in phosphate-buffered saline/casein and once in 50 mM-Tris/HCl, pH 7.6. The staining reaction was done with 5 mg of diaminobenzidine and 3.3 μl of H2O2 (30%) in 10 ml of 50 mM-Tris/HCl (pH 7.6).

Gel filtration

High performance gel filtration was performed on a TSK 3000 column (LKB, Bromma, Sweden).
equilibrated with phosphate-buffered saline (pH 6.8). Preparation of the samples and other details are given in the legend to Fig. 5.

**Solubilization of dipeptidylpeptidase IV from crossed immunoelectrophoresis immunoprecipitates**

Immunoprecipitates of dipeptidylpeptidase IV were excised from the wet gel under dark-field illumination, suspended by sonication in 2 ml of 8 M-urea and left for 2 h at room temperature. Full enzymic activity was recovered after this treatment. Pansorbin beads (100 μl of a 10% suspension) were added and the solution was dialysed with continuous stirring against 2 mM-Tris/HCL (pH 7.0) containing 0.1% Berol. After centrifugation at 10,000 g for 10 min, 90% of the dipeptidylpeptidase IV activity was recovered in the supernatant, while 10% remained associated with the beads.

**Enzymic activity**

Samples of the solubilized antigens (see above) were made 50 mM in Tris/HCL (pH 7.8), containing 50 μM-bestatin. After 2 h at 37°C, the solution was made 1 mM in Gly-Pro-β-NNap (or Ala-Ala-β-NNap) (final vol. 300 μl) and dipeptidylpeptidase IV activity was determined spectrophotometrically by measuring the rate of liberation of β-naphthylamine from the amino acid derivatives at 340 nm. In these conditions the rate of substrate hydrolysis increased linearly as a function of time and enzyme concentration. Absorbance values were corrected for turbidity and spontaneous substrate hydrolysis. Enzyme activity is expressed as nmol of substrate hydrolysed/min using the absorption coefficient 1780 m⁻¹·cm⁻¹.

**Results**

**Visualization of dipeptidylpeptidase IV in crossed immunoelectrophoresis**

Crossed immunoelectrophoresis of normal human fibroblasts against an oligospecific anti-human fibroblast) serum (aNHFiv), obtained by intravenous injection of rabbits with whole cells, revealed one major double precipitate, identified previously as aminopeptidase M (Verlinden *et al.*, 1981c). When cell extracts were incubated at pH 5.5 the slow migrating peak of aminopeptidase was converted into the fast-migrating precipitate. In addition, another sharp peak of slower mobility than, and intersecting with, the aminopeptidase precipitate became visible (Fig. 1b). Alkalination of the acid extract (to pH 8.6) did not restore the pattern observed with pH 8.6 cell extracts, suggesting that acidification irreversibly altered the antigen. Specific antibodies against this newly visualized antigen were obtained by injecting rabbits with the immunoprecipitate, excised from unstained crossed immunoelectrophoresis plates. Crossed immunoelectrophoresis of an alkaline cell extract (pH 8.6) with this antiserum resulted in a broad asymmetric precipitate, with β-mobility (Fig. 1c). In an acid cell extract (pH 5.5) the peak was sharper and of α-mobility (Fig. 1d). Increased mobility in crossed immunoelectrophoresis after acid extraction, probably due to endogenous proteolysis, was described for aminopeptidase M (Verlinden *et al.*, 1981c). Also, membrane hydrolases of kidney and intestine were shown to have an increased mobility in crossed immunoelectrophoresis after proteinase, compared with detergent extraction (Kenny & Maroux, 1982). Identification of the immunoprecipitates through enzymic staining with different substrates specific for brush border hydrolases was therefore attempted. Isomaltase, sucrase, maltase, aminopeptidase or neutral endopeptidase activity could not be demonstrated in the immunoprecipitates. Gly-Pro-β-NNap,
Table 1. Dipeptidylpeptidase IV activity in cell extracts

The equivalent of 1.6 x 10⁷ intact trypsinized NHF fibroblasts, the supernatant of the same amount of sonicated cells centrifuged at 130000g for 6 min and the corresponding pellet solubilized in 2% Berol were tested for enzyme activity towards Gly-Pro-β-NNap. The samples were diluted in 50 mM-Tris/HCl (pH 7.8), containing 1 mM-Gly-Pro-β-NNap, and the change in A₃₄₀ was measured spectrophotometrically at 37°C. Protein concentration was determined according to Lowry et al. (1951). The mean (± S.E.M.) of four determinations is given.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Total activity (nmol/min)</th>
<th>Specific activity (nmol/min per mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact cells</td>
<td>420 ± 34</td>
<td>562 ± 28</td>
</tr>
<tr>
<td>Supernatant</td>
<td>96 ± 11</td>
<td>225 ± 39</td>
</tr>
<tr>
<td>Pellet</td>
<td>348 ± 34</td>
<td>1388 ± 180</td>
</tr>
</tbody>
</table>

A substrate specific for dipeptidylpeptidase IV, however, gave a clear staining of the peak (Figs. 1e and 2). In crossed immunoelectrophoresis against a polyspecific anti-fibroblast serum (aNHF) generating more than 20 visible peaks (Verlinden et al., 1981a) only the peak under investigation stained enzymically with Gly-Pro-β-NNap (results not shown).

Membrane localization of dipeptidylpeptidase IV

Dipeptidylpeptidase IV was labelled metabolically by [³⁵S]methionine and [¹⁴C]glucose (results not shown), indicating that it was a cellular glycoprotein. Moreover, the antigen was labelled by exogenous lactoperoxidase-catalysed ¹²⁵I iodination of living cells, suggesting its exposure at the cell surface (results not shown). Quantification of the enzyme activity in intact cells and in cell extracts indicated that the 130000g pellet of fibroblasts, sonicated in the absence of detergent, contained 78% of the recovered activity (Table 1). The enzyme activity could only be solubilized from the pellets by detergent extraction. This was confirmed in crossed immunoelectrophoresis, since the peak corresponding to dipeptidylpeptidase IV was only observed after incubation of the 130000g pellets of sonicated cells with 2% Berol. In addition, the dipeptidylpeptidase IV peak with a mobility obtained by acid-detergent extraction did not appear when detergent was omitted, even after 18 h incubation at pH 5.5 and 37°C. Treatment of cell extracts with papain gave comparable results. Fig. 2 illustrates the gradual shift in mobility from β to α of detergent-solubilized dipeptidylpeptidase IV exposed to increasing papain concentrations. A similar papain treatment in the absence of detergent did not solubilize dipeptidylpeptidase IV and subsequent addition of 2% Berol to the resuspended cell pellets yielded a peak which still migrated at β mobility in crossed immunoelectrophoresis (results not shown).

Finally, hydrophobic-interaction crossed immunoelectrophoresis was used to examine whether the antigen contained a hydrophobic domain. Phenyl-Sepharose beads were incorporated in the first-dimension gel of crossed immunoelectrophoresis (Bjerrum, 1978). Detergent-solubilized dipeptidylpeptidase IV was completely retained (Fig. 3a). Even during the second dimension electrophoresis in the presence of detergent, the dipeptidylpeptidase IV molecules were only partially detached from the hydrophobic beads. The acid-extracted α-peak, however, was minimally retarded (Fig. 3b).

M₄ determinations

Immunoprecipitates of the acid-extracted α- and the detergent-solubilized β-forms of dipeptidylpeptidase IV, from ¹²⁵I/lactoperoxidase-labelled fibroblasts, were excised from unstained crossed immunoelectrophoresis gels and applied to parallel slots of a 6–20% polyacrylamide gradient slab gel (Fig. 4, lanes a and b). The autoradiograms showed that both forms of dipeptidylpeptidase IV consist of different subunits with apparent M₄ values of 125000 and 135000. The large subunit of the α-form consistently showed a slightly higher mobility.
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Fig. 3. Hydrophobic interaction crossed immunoelectrophoresis

The detergent-solubilized 130000 g pellet of NHF fibroblasts, which had been sonicated in the absence of Berol (approx. 10⁶ cells) (a) and fibroblasts extracted in 2% Berol at pH 5.5 for 2h at 37°C (b) were run against anti-(dipeptidylpeptidase IV). Phenyl–Sepharose beads were included in the first-dimension gel.

Fig. 4. SDS/polyacrylamide-gel electrophoresis

(a) and (b) are autoradiograms of 6–20% gradient slab gels of ¹²⁵I-labelled peaks of dipeptidylpeptidase IV excised from wet crossed immunoelectrophoresis plates. In (a) the detergent-solubilized form of dipeptidylpeptidase IV was applied; (b) contains the acid-extracted form. Lanes (c) and (d) were transferred to nitrocellulose by blotting; the detergent-solubilized form of dipeptidylpeptidase IV, excised from crossed immunoelectrophoresis plates, was applied, respectively treated with (c) and without (d) 5% 2-mercaptoethanol. Cold-insoluble globulin (M, 220000), β-galactosidase (M, 135000 and 67000), RNA polymerase (M, 165000, 155000 and 39000) and ovalbumin (M, 45000) were used as markers.

(lane b) than did the corresponding subunit of the β-form (lane a). Immunostaining on nitrocellulose replicas of the 6–20% gradient polyacrylamide gel (see the Experimental section) also revealed the two bands (Fig. 4, lane c). When treatment with 2-mercaptoethanol was omitted, however, the enzyme barely entered the gel (Fig. 4, lane d). In a 4–15% polyacrylamide gradient gel, unreduced dipeptidylpeptidase IV migrated somewhat slower than α₂-macroglobulin half-molecules (M, 360000) used as marker (results not shown).

The native M, of the enzyme was determined by high performance gel filtration of an acid cell extract containing dipeptidylpeptidase IV in the α-form and of a detergent extract which yields a dipeptidylpeptidase IV peak at the β position in crossed immunoelectrophoresis. Elution of dipeptidylpeptidase IV, monitored by measuring enzyme activity, corresponded to an M, of 400000 in both preparations (Fig. 5). An additional peak of activity in the high-molecular-weight range was observed for the detergent-extracted preparation.

Enzyme activity of the different forms of dipeptidylpeptidase IV

To determine the enzyme activity of dipeptidylpeptidase IV with different mobilities in crossed immunoelectrophoresis, the immunoprecipitates were excised from crossed immunoelectrophoresis agarose gels and dissociated in 8M-urea (2h at room temperature). Kidney dipeptidylpeptidase IV was shown to be stable under these conditions for at least 4h (Barth et al., 1974) and similarly we found no decrease in enzyme activity on fibroblast dipeptidylpeptidase IV by this treatment. Svensson et al. (1978) observed that intestinal dipeptidylpeptidase IV was eluted from immobilized specific antibodies with 2mm-Tris/HCl buffer (pH 7). We therefore dialysed the 8M-urea preparations against this buffer in order to dissociate the immune complexes. An excess of Pansorbin beads, added to remove IgG and any remaining antigen–antibody complexes, bound only 10% of the total dipeptidylpeptidase IV activity. In SDS/polyacrylamide-gel electrophoresis the two characteristic bands were observed for both forms of the solubilized antigen. The hydrolysis of two substrates, Gly-Pro-β-NNap and Ala-Ala-β-NNap, specific for kidney dipeptidylpeptidase IV, was measured. Since the presence of small amounts
of aminopeptidase would influence the measurements, both by stepwise cleavage of Ala-Ala-β-NNap (Barth et al., 1974) or by altering the kinetics of dipeptidylpeptidase IV activity (Wolf et al., 1978), the specific aminopeptidase inhibitor bestatin (Umezawa et al., 1976) was added to the test medium. In agreement with Hazato et al. (1982) we found that 50μM-bestatin had no influence on dipeptidylpeptidase IV activity. Samples of both the acid-extracted α form and the alkaline-solubilized β form of dipeptidylpeptidase IV hydrolysed Ala-Ala-β-NNap 10 times more slowly than Gly-Pro-β-NNap. The sensitivity of the Gly-Pro-β-NNap hydrolysing activity of dipeptidylpeptidase IV to di-isopropylphosphorofluoridate was also measured. Fractions of the acid extracted α form of dipeptidylpeptidase IV were preincubated for 90 min with different di-isopropylphosphorofluoridate concentrations in 50 mM-Tris/HCl (pH 7.8) at 37°C. Then Gly-Pro-β-NNap was added to a final concentration of 1 mM. The rate of hydrolysis of the control sample was 187 nmol/min; 50% inhibition was obtained with 0.023 mM-di-isopropylphosphorofluoridate.

**Discussion**

Hopshu-Hau & Ekfors (1969) have shown that dermal fibroblasts, especially those located around hair follicles, display high dipeptidylpeptidase IV activity, while human skin represented only a minor source of dipeptidylpeptidase IV activity compared with other tissues (Hopshu-Hau & Ekfors, 1969; De Bersaques, 1970). We identified dipeptidylpeptidase IV in human fibroblasts by direct enzymic staining of the crossed immunoelectrophoresis gels with Gly-Pro-β-NNap. The solubilized antigen had similar enzymic properties to the well-characterized pig kidney dipeptidylpeptidase IV (Barth et al., 1974; Kenny et al., 1976). The molecule was membrane-associated, located at the outer cell-surface and it contained a hydrophobic domain. In the presence of detergent the hydrophobic domain was lost upon acid treatment at pH 5.5, also resulting in an increased mobility in crossed immunoelectrophoresis. This was probably due to the action of lysosomal enzymes. Papain treatment had a similar effect. Macnair & Kenny (1979) and Booth & Kenny (1980) described an Mr 4000 hydrophobic anchor for kidney dipeptidylpeptidase IV. The fibroblast dipeptidylpeptidase IV hydrophobic domain must also be small since no major difference in Mr value was observed between acid- and detergent-solubilized molecules in gelfiltration. In SDS/polyacylamide gel electrophoresis after reduction, only the subunit of Mr 135 000 appeared to migrate somewhat faster.

Dipeptidylpeptidase has been isolated from different tissues such as rat liver (Hopshu-Hau & Sarino, 1976), human submaxillary gland (Oya et al., 1972), pig kidney (Barth et al., 1974; Kenny et al., 1976), lamb kidney (Yoshimoto & Walter, 1977), human kidney (Hama et al., 1982), pig intestine (Svensson et al., 1978), pig pancreas (Yoshimoto et al., 1982) and human placenta (Püschel et al., 1982). All these molecules displayed a native Mr between 220 000 and 280 000. It was therefore surprising to find that human fibroblast dipeptidylpeptidase IV had an Mr of 400 000 in gel filtration. In addition, the enzyme is apparently composed of different subunits (Mr 125 000 and Mr 135 000) linked by disulphide bonds. This again contrasts with the enzymes previously described (see above) since these were dissociated, by denaturation only, into two subunits with an identical Mr in the range of 110 000–137 000. The high-molecular-weight peak seen in gel filtration of the detergent-solubilized β form of dipeptidylpeptidase IV is probably due to formation of lipid–detergent micelles.

It is interesting to note that an Mr 400 000 peak, with dipeptidylpeptidase IV activity, was seen in gel filtration of human urine (Kato et al., 1978). Preliminary results indicate that this molecule is at...
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least immunologically related to fibroblast dipeptidylpeptidase IV, since antibodies directed against human urine proteins precipitate dipeptidylpeptidase IV from fibroblast cell extracts in crossed immunoelectrophoresis (M. Saison, J. Verlinden, F. Van Leuven, J.-J. Cassiman & H. Van den Berghe, unpublished results).

In conclusion, our investigations demonstrated the presence of dipeptidylpeptidase IV activity at the cell membrane of human fibroblasts. Together with aminopeptidase M (Verlinden et al., 1981c), it is the second N-terminal peptidase present in large amounts. Whether the occurrence of these enzymes is important for a particular activity of fibroblasts remains to be determined.

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References