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A collagen-binding glycoprotein on the surface of mouse fibroblasts is identified as dipeptidyl peptidase IV

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A dipeptidyl aminopeptidase (DPP) was detected in plasma membranes from normal (3T3) and transformed (3T12) mouse fibroblasts. This enzyme was active in cleaving the prolyl bond in the synthetic dipeptide nitroanilide Gly-Pro-NH-Np, which is a specific substrate for DPP IV (K_m 0.63 mM and $V_{max.}$ 6.1 nmol/min per mg at pH 6.0 and 37 °C). However, it did not degrade Pro-NH-Np or other dipeptide nitroanilides such as Gly-Arg-NH-Np or Val-Ala-NH-Np. The enzyme was totally inhibited by di-isopropyl phosphorofluoridate (Pr^i_2-*P*-F) and by phenylmethanesulphonyl fluoride, indicating a serine catalytic site for the proteinase. DPP IV is a glycoprotein that specifically recognized immobilized gelatin and type I collagen. Upon molecular exclusion chromatography, the proteinase exhibited an apparent M_r of 100000. SDS/polyacrylamide-gel electrophoresis under non-reducing and reducing conditions revealed that the [^{3}H]Pr^i_2-*P*-protein was exclusively represented by a polypeptide of M_r 55000. This suggested that DPP IV consists of two non-covalently linked 55000-M_r subunits. Fibroblast adhesion to native or denatured collagen was significantly inhibited by the two dipeptide inhibitors of DPP IV, Gly-Pro-Ala and Ala-Pro-Gly, but not by the peptides Gly-Pro and Gly-Gly-Gly, which are not inhibitors of the proteinase. Moreover, preliminary fractionation of DPP IV by molecular exclusion chromatography and affinity chromatography indicated that this material was active in disrupting cell adhesion to collagens. Taken together, the above data suggest that a fibroblast membrane-associated collagen-binding glycoprotein, DPP IV, may play a role in cell attachment to collagen.

INTRODUCTION

Several proteins such as fibronectin, laminin and collagens contribute to the structural integrity of the extracellular matrix and thus may be involved in cell attachment, migration and growth control. Cell-surface receptors for fibronectin and laminin participating in cell attachment have been reported in several cell types (Lesot *et al.*, 1983; Malinoff & Wicha, 1983; Rao *et al.*, 1983; Horwitz *et al.*, 1985; Pytela *et al.*, 1985; Akiyama *et al.*, 1986; Orushihara & Yamada, 1986). Cell–collagen interactions may occur via fibronectin or laminin, which act as mediator proteins by forming bridges between specific membrane receptors and collagen (Kleinman *et al.*, 1981; Woodley *et al.*, 1983; Yamada, 1983; Charonis *et al.*, 1985). However, adhesion mechanisms independent of such bridging proteins have also been demonstrated with respect to the adhesion of some cell lines to collagen (Grinnell & Minter, 1978; Schor & Court, 1979; Aumailley & Timpl, 1986; Bauvois & Roth, 1987), suggesting that receptor–ligand type of interactions operate in cell–collagen adhesion phenomena. Various membrane protein receptors with affinity for different types of collagen have been identified, although it is not known whether these components participate in cell attachment (Koehler *et al.*, 1980; Chiang & Kang, 1982; Lesot *et al.*, 1983; Mollenhauer & Von der Mark, 1983; Kurkinen *et al.*, 1984; Kotite & Cunningham, 1986; Dedhar *et al.*, 1987). By using collagen affinity-chromatographic and immunological approaches workers in two laboratories have demonstrated the ability of antibodies directed against cell-surface collagen-binding protein to perturb cell adhesion to insolubilized collagen. These collagen-binding proteins were isolated from chondrocytes (anchorin CII, M_r 31000, specific of collagen II) (Mollenhauer *et al.*, 1984) and rat hepatocytes (complex of three proteins, M_r 160000, 140000 and 130000, interacting with collagen I) (Rubin *et al.*, 1986).

The central feature of collagens is their repeating sequences with the general structure Gly-Pro-Xaa (Piez *et al.*, 1963). Two peptidase activities capable of releasing Gly-Pro or Gly-Pro-Xaa from chromogenic derivatives were seen as having the potential for playing a role in the breakdown of collagen. The tripeptide-releasing aminopeptidase named tripeptidyl peptidase I was identified in ovary lysosomes from pregnant pigs (McDonald *et al.*, 1985). Dipeptidyl peptidase IV (DPP IV) that specifically removes dipeptides of the Gly-Pro type was detected in serum and in a variety of tissues (for a review see Kreisel *et al.*, 1982; Hanski *et al.*, 1985). DPP IV was first isolated from rat liver plasma membrane and identified as a sialoglycoprotein consisting of two identical disulphide-linked subunits of M_r 110000–120000 (Elovson, 1980; Kreisel *et al.*, 1980, 1982). More recently, Hanski *et al.* (1984) reported the presence of similar peptidase in mouse liver plasma membranes with an M_r of 105000. Moreover, the antibody directed against mouse liver plasma-membrane DPP IV cross-reacted with the rat liver enzyme but did not recognize the

Abbreviations used: DPP, dipeptidyl peptidase; Bz-, benzoyl-; Z-, benzyloxycarbonyl-; Hip-, *N*-benzoylglycyl-; -NH-Np, *p*-nitroanilide; -NH-(MeO)Nap, β-4-methoxynaphthylamide; Pr^i_2-*P*-F, di-isopropyl phosphorofluoridate.

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enzyme on the surface of hamster fibroblasts (Hanski *et al.*, 1985). Cell–matrix interactions studies conducted on baby-hamster kidney cells and on rat hepatocytes, grown on denatured collagen in the presence of fibronectin, suggested that DPP IV of these cells may be involved in fibronectin-mediated cell spreading on collagen (Hanski *et al.*, 1985).

In the present study, I have identified a cell-surface-associated 100000-M_r glycoprotein from mouse fibroblasts that binds type I collagen and is a DPP IV. Its possible involvement in the adhesion of mouse fibroblasts to collagen is considered.

EXPERIMENTAL

Materials

^{125}I-labelled Bolton–Hunter reagent and [^{3}H]di-isopropyl phosphorofluoridate ([^{3}H]Pr^i_2-*P*-F) were obtained from New England Nuclear. Unlabelled Pr^i_2-*P*-F, Leu-NH-Np, Z-Pro-Ala-Gly-Pro-NH-(MeO)Nap, Ala-Pro-Gly, Gly-Pro, Gly-Arg-NH-Np, Gly-Pro-Ala, Gly-Gly-Gly, Val-Ala-NH-Np, Hip-L-Arg and Hip-L-Phe were all purchased from Serva. The other synthetic substrates, the proteinase inhibitors, gelatin–Sepharose, bovine serum albumin–Sepharose, casein–Sepharose and all other chemicals were supplied by Sigma Chemical Co. Ultrogel AcA 44 was purchased from IBF (Villeneuve la Garenne, France) and n-octyl β-D-glucoside was from Boehringer, The lectin affinity gels and Sepharose 4B were products of Pharmacia Fine Chemicals. Calibration proteins for gel electrophoresis and silver stain kit were acquired from Bio-Rad Laboratories. Collagen (type I, acid-soluble, from calf skin) was coupled to Sepharose 4B according to the procedure of Hannan & McAuslan (1983). MP hyperfilms were from Amersham International.

Cells

Balb/c 3T3 and Balb/c 3T12 cells were grown in RPMI 1640 medium (Flow Laboratories) supplemented with 25 mM-Hepes, pH 7.4, 10% (v/v) fetal-calf serum, 2 mM-glutamine and 50 μg of gentamicin/ml. For subculturing and harvesting, confluent layers of cells were treated with 0.01% trypsin as previously described (Bauvois & Roth, 1987).

Cell-surface iodination

Harvested cells were washed twice by resuspension and centrifugation in 10 mM-Tris/HCl buffer, pH 7.4, containing 0.154 M-NaCl. Radioiodination of cells with ^{125}I-labelled Bolton–Hunter reagent was carried out on ice by addition of the radioisotope (1 mCi) to the cell suspension (5×10^7 cells/0.5 ml). The reaction was terminated after 15 min by dilution with ice-cold Tris/HCl buffer, pH 7.4, containing 0.154 M-NaCl and 1 M-glycine, followed by three rinses of the labelled cells with this buffer. The cell pellet was lysed in 1% (w/v) octyl β-D-glucopyranoside (in 10 mM-Tris/HCl buffer, pH 7.4) (2×10^7 cells/ml) and subsequently centrifuged at 1000 *g* for 10 min. The resulting supernatant was denoted the detergent extract.

Membrane preparation

Plasma membranes were purified from freshly harvested cells or from washed cells stored at −20 °C. All steps were performed at 4 °C. Washed cells (1×10^7–5×10^7) were resuspended in 4 ml of 0.25 M-sucrose in 10 mM-Tris/HCl buffer, pH 7.4, containing 0.2 mM-$MgCl_2$ and 5 mg of bovine serum albumin/ml and were submitted to four cycles of freezing–thawing, which was followed by five strokes with a rotating Potter–Elvehjem homogenizer. Total cell lysis was monitored by measuring supernatant lactate dehydrogenase activity and compared with that from 100%-detergent-lysed cells. The final material was centrifuged at 1000 *g* for 10 min to remove the nuclear pellet. The supernatant was centrifuged at 40000 *g* for 30 min, and the resulting pellet was resuspended in 3 ml of 0.25 M-sucrose solution and layered on 8 ml of 1 M-sucrose solution in 10 mM-Tris/HCl buffer, pH 7.4, containing 0.2 mM-$MgCl_2$ and 5 mg of bovine serum albumin/ml. The resulting gradient was centrifuged at 100000 *g* for 1 h. The membrane fraction was collected in the 0.25 M-/1 M-sucrose interface (Massague & Czech, 1982; Massague, 1983), diluted with 10 mM-Tris/HCl buffer, pH 7.4, containing 0.154 M-NaCl and 0.2 mM-$MgCl_2$ and centrifuged at 39000 *g* for 30 min. This membrane fraction was found to be devoid of glucuronidase and to possess less than 1% of lactate dehydrogenase and *N*-acetyl-β-D-glucosaminidase activities. Solubilization of pelleted membranes was performed by homogenization of the pellet in 10 mM-Tris/HCl buffer, pH 7.4, containing 1% (w/v) n-octyl β-D-glucoside (1 mg of membrane protein per ml of solubilization buffer).

Assay for DPP IV activity

DPP IV activity was routinely assayed by incubating 20 μg of plasma membranes (or enzyme fraction) for 4 h at 37 °C with 0.5 mM-Gly-Pro-NH-Np in 10 mM-sodium citrate buffer, pH 6.0, containing 0.154 M-NaCl (final volume of the incubation mixture 0.5 ml). A control mixture lacking plasma membranes was also tested. The activity was determined from the amount of *p*-nitroaniline formed (absorbance increase at 410 nm) and quantified by reference to a standard curve prepared with *p*-nitroaniline (Hanski *et al.*, 1985). The effect of pH on DPP IV activity was examined at 37 °C for 4 h with assay mixtures containing 2 mM-Gly-Pro-NH-Np; 10 mM-sodium citrate buffer containing 0.154 M-NaCl was used in the pH range 3.1–6, 10 mM-sodium phosphate buffer containing 0.154 M-NaCl in the pH range 5.5–7.1, 10 mM-Tris/HCl buffer containing 0.154 M-NaCl in the pH range 7.1–8 and 10 mM-sodium tetraborate buffer containing 0.154 M-NaCl in the pH range 7.8–9.8. For testing the effect of potential inhibitors, enzyme/pH 6.0 buffer mixtures and inhibitor were preincubated for 30 min (proteinase inhibitors) or 15 min (di- and tri-peptides) at 37 °C. Remaining activity was measured by adding Gly-Pro-NH-Np to a final concentration of 0.5 mM and the reaction was conducted for 4 h. Control mixtures lacking enzyme or inhibitor were also tested. Remaining activity is expressed as percentage of the control activity (without inhibitor).

Other enzyme assays

To characterize the proteinases in the membrane preparations, activity measurements with a number of chromogenic substrates were performed at various pH values. Leu-NH-Np, Hip-Phe and Bz-Arg-NH-Np were dissolved in dimethyl sulphoxide. The solvent had no effect in the range of dilution used. Trypsin-like proteinase activity was assayed on *p*-tosyl-L-arginine

methyl ester by measuring the increase in absorbance at 247 nm resulting from the liberation of *p*-tosyl-L-arginine (Rick, 1974). Amidase activity was determined on Bz-DL-arginine-NH-Np (Fritz *et al.*, 1974), aminopeptidase activity was assayed on Leu-NH-Np, and dipeptidyl aminopeptidase activities were assayed on Gly-Arg-NH-Np and Val-Ala-NH-Np. Formation of *p*-nitroaniline was recorded at 410 nm. Endo-prolyl peptidase activity was determined on the naphthylamide Z-Pro-Ala-Gly-Pro-NH-(MeO)Nap. The reaction was monitored by measuring the change in absorbance at 340 nm. Carboxypeptidase A and B activities were determined by measuring the increase in absorbance at 254 nm resulting from the liberation of hippuric acid with Hip-L-Phe and Hip-L-Arg respectively as the substrates (Appel, 1974; Bergmeyer *et al.*, 1974).

Ultrogel AcA 44 chromatography

Detergent-solubilized membrane components (200–500 μg of protein) were subjected to molecular exclusion chromatography on an Ultrogel AcA 44 column (0.5 cm × 35 cm) (fractionation range M_r 10000–130000) equilibrated with 10 mM-citrate buffer, pH 6.0, containing 1 M-NaCl and 0.001 % n-octyl β-D-glucoside. Elution was conducted at 4 °C, and 0.25 ml fractions were collected at a flow rate of 14 ml/h. Protein absorbance was monitored at 280 nm and proteolytic activity was measured for 20 h as described above. The apparent M_r of DPP IV was determined from a calibration curve of $K_{av.}$ plotted against log M_r for protein standards.

Affinity chromatography

The purification scheme performed at 4 °C was the same for gelatin–Sepharose (5 ml) and collagen–Sepharose (5 ml). Before each use the affinity matrix was washed with 4 M-urea to remove residual bound proteins. The octyl glucoside extract (500 μg of plasma proteins) was applied to the affinity matrix equilibrated with 10 mM-Tris/HCl buffer, pH 7.4, containing 0.001 % octyl glucoside (buffer A). The column was washed with the same buffer, then eluted with 0.154 M-NaCl in buffer A and finally with 1 M-NaCl in buffer A. Fractions (1 ml) were collected and the absorbance was monitored at 280 nm. The fractions containing proteolytic activity were pooled.

[^{3}H]Pr$^i_2$-*P*-F labelling of DPP IV

Samples were incubated for 30 min at 37 °C with [^{3}H]Pr$^i_2$-*P*-F (4.7 Ci/mmol) at a final concentration of 0.1 mM in 10 mM-citrate buffer, pH 6.0, containing 0.154 M-NaCl. After incubation, proteins were precipitated with 6 vol. of cold acetone, kept overnight at −20 °C and recovered by centrifugation and analysed by SDS/polylacrylamide-gel electrophoresis.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis in the presence of 0.1 % SDS was carried out in a 7.5 % gel or in a 7–12 % gel gradient (Laemmli, 1970) under non-reducing and reducing conditions. Samples were precipitated with acetone at −20 °C and dissolved in sample buffer at 90 °C for 15 min. Reduction was carried out by adding 2-mercaptoethanol to a final concentration of 5 % in the sample buffer. The M_r markers consisted of myosin (200000), β-galactosidase (116000), phosphorylase *b* (94000), bovine serum albumin (66500), ovalbumin (43000) and carbonic anhydrase (31000). Proteins were detected by $AgNO_3$ staining as described by Bio-Rad Laboratories, a method derived from that of Merril *et al.* (1981). ^{125}I-labelled proteins were detected by placing the dried gel against an Amersham MP Hyperfilm plus an intensifying screen. For detection of [^{3}H]-labelled proteins the gel was soaked 3 min in Amplify Amersham, dried and exposed to an Amersham HP Hyperfilm at −70 °C.

Protein determination

Protein was determined by the method of Geiger & Bessmann (1972), with bovine serum albumin as the standard.

Cell-adhesion assays

The adhesion of 3T12 cells to collagens and fibronectin was assayed as previously described (Bauvois & Roth, 1987). Briefly, denatured collagen or native collagen was co-polymerized with glutaraldehyde in micro-titre wells (24 flat-bottom wells with a capacity of 2.8 ml). Fibronectin substrata were prepared by overnight incubation of plasma fibronectin in micro-titre wells previously coated with denatured collagen. Harvested cells (1×10^5–2.5×10^5) were resuspended in 200 μl of adhesion medium (10 mM-Tris/HCl buffer, pH 7.4, containing 0.154 M-NaCl and 5 mM-$MnCl_2$) and were added to the micro-titre well at 37 °C. At the end of the incubation, the non-attached cells were removed with two 500 μl rinses of adhesion medium minus $MnCl_2$. Lactate dehydrogenase content was used to quantify adhering cells. Cell adhesion was expressed as the percentage of total cells adhering to each well. The effects of soluble di- and tri-peptides were tested by preincubating the cells with the effector for 15 min at room temperature, followed by transfer of the cell suspension to the micro-titre well. The effect of purified DPP IV was tested by preincubating for 30 min at room temperature the insolubilized substrate with the 0.001 %-detergent-solubilized DPP IV (100 μl) before addition of 3T12 cells. Adhesion was then measured after 10 min on collagens and 5 min on fibronectin (this short-time adhesion corresponded to the affinity-dependent binding of cells to the substrata, according to Bauvois & Roth, 1987).

RESULTS

Identification of a DPP IV associated with fibroblast membranes

Preliminary findings showed that 3T3-cell and 3T12-cell homogenates possess an activity for degradation of Gly-Pro-NH-Np at pH 7.4. The presence of this enzyme in purified plasma membranes of 3T3 and 3T12 fibroblasts was investigated. Both normal and transformed cells showed high activity towards Gly-Pro-NH-Np (1.19 ± 0.45 μmol/4 h per mg and 0.70 ± 0.16 μmol/4 h per mg for 3T3 and 3T12 cells respectively; means of five experiments). Inactivity with Pro-NH-Np and with Z-Pro-Ala-Gly-Pro-NH-(MeO)Nap indicated that the hydrolysis of Gly-Pro-NH-Np is not due to a proline-specific endopeptidase. Moreover, experiments with two other dipeptide *p*-nitroanilide derivatives Gly-Arg-NH-Np and Val-Ala-NH-Np, showed no cleavage of *p*-nitroaniline, again indicating that fibroblast membranes possess a specific exopeptidase that preferentially cleaves prolyl bonds in Gly-Pro sequences. When

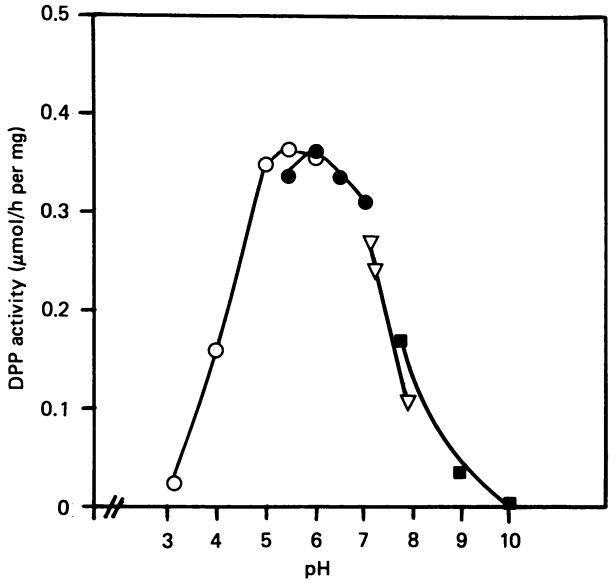

Fig. 1. Effect of pH on the rate of hydrolysis of Gly-Pro-NH-Np by DPP IV from solubilized 3T3-cell plasma membranes

Solubilized plasma membranes were incubated with 2 mM-Gly-Pro-NH-Np for 4 h at 37 °C at different pH values: 10 mM-citrate buffer containing 0.154 M-NaCl were used in the pH range 3.1–6.0 (○), 10 mM-phosphate buffer containing 0.154 M-NaCl in the pH range 5.5–7.1 (●), 10 mM-Tris buffer containing 0.154 M-NaCl in the pH range 7.1–8.0 (▽) and 10 mM-sodium tetraborate buffer containing 0.154 M-NaCl above pH 7.8 (■). Rates were determined as described in the Experimental section.

the membrane preparations were examined for the presence of other proteinases, no activity was demonstrated on Bz-Arg-NH-Np and *p*-tosyl-L-arginine methyl ester, which are the typical substrates of trypsin-like proteinases. No detectable activity was found with the substrates Hip-Phe and Hip-Arg, which are normally used to measure carboxypeptidase A and B activities. However, the membranes also appeared to possess a high aminopeptidase activity (about 5.2 μmol/4 h per mg) that hydrolysed Leu-NH-Np. It has previously been shown that DPP IV is found in serum (Hino *et al.*, 1976). In our conditions, contamination of the plasma-membrane preparations with fetal-calf serum, in the presence of which the cells are grown, is unlikely, because no activity of serum DPP IV was detectable in the range of dilution used corresponding to the possible contamination.

Characterization of DPP IV activity

The rate of hydrolysis of Gly-Pro-NH-Np by the plasma membranes was time-dependent with a linear relationship up to 20 h. Moreover, the initial rate of the reaction was dependent on the amount of plasma-membrane DPP IV (up to 40 μg of protein). The activity of the enzyme was tested over the pH range 3–9. As shown in Fig. 1, activity was maximal in the pH range 5–6 with citrate buffer and at pH 6.0 with phosphate buffer. A Lineweaver–Burk plot gave a K_m of 0.63 mM, and a $V_{max.}$ of 6.1 nmol/min per mg was determined for Gly-Pro-NH-Np at pH 6.0 and at 37 °C.

The effects of various potential inhibitors on enzyme activity are summarized in Table 1. Among the compounds tested, Pr^i_2-*P*-F and phenylmethanesulphonyl fluoride totally inactivated Gly-Pro-NH-Np hydrolysis, providing evidence for a serine residue at the active site of DPP IV. Soya-bean trypsin inhibitor, a trypsin-like-proteinase inhibitor, was without effect. Thiol-reactive compounds such as iodoacetate, *N*-ethylmaleimide and dithioerythritol had no inhibitory effect on the activity of the enzyme. However, its apparent inhibition by 2-mercaptoethanol suggested the presence of essential disulphide bond(s) in the molecule. Moreover, leupeptin and pepstatin, two potent inhibitors of endopeptidase, were ineffective. Finally, the enzyme activity was not affected by EDTA or by bivalent cations such Mn^{2+}, Mg^{2+} and Ca^{2+} up to 5 mM. In conclusion, it appears that DPP IV is a serine peptidase that requires one or several disulphide bonds in the molecule for its activity. The presence of these disulphide bonds may reflect the high stability of the enzyme (no loss in activity was observed when the plasma membranes were stored for several months at −20 °C or frozen–thawed several times).

Table 1. Effects of potential inhibitors on DPP IV

Relative activity is expressed as the percentage of that obtained without added inhibitor. Preincubation of inhibitors and enzyme was done at 37 °C for 30 min, before addition of 0.5 mM-Gly-Pro-NH-Np. Incubation assays were conducted for 4 h at 37 °C and at pH 6.

Inhibitor	Concn. (mM)	Relative activity (%)
None		100
Pr^i_2-*P*-F	0.1	2
	0.5	0
Phenylmethanesulphonyl fluoride	0.1	39
	0.5	2
Soya-bean trypsin inhibitor	0.1	95
Iodoacetate	2	108
N-Ethylmaleimide	2	124
Dithioerythritol	2	115
2-Mercaptoethanol	0.5	49
	1	27
Leupeptin	2	108
Pepstatin	2	131

Isolation of DPP IV

The results of a typical purification of the enzyme from 3T12 fibroblasts are summarized in Table 2. The elution profile in 1 mM-NaCl of solubilized membrane proteins from a molecular exclusion chromatography on Ultrogel AcA 44 is shown in Fig. 2. The void volume contained mainly proteins of M_r above 130000 (aggregated or not). DPP IV activity, however, was eluted mainly in a second peak (85%) in the region of M_r 100000 (as estimated by calibration of the column with standard proteins). No DPP IV activity was found in the lower-M_r ranges. At this step about 40% of the input total DPP IV activity was lost during the fractionation. Moreover, leucine aminopeptidase activity detected in the membrane preparation, by contrast with DPP IV activity, was eluted in the exclusion peak of the column. Affinity

Table 2. Partial purification of DPP IV from mouse fibroblasts

For determination of DPP IV activity, incubations were carried out for 4 h with 1 mM-Gly-Pro-NH-Np and a sample of the fraction to be tested, at pH 6 and at 37 °C.

Purification step	Protein (μg)	Activity (μmol/4 h)	Specific activity (μmol/4 h per mg)	Yield (%)	Purification factor
Solubilized membranes	203	0.14	0.69	100	1
100000-M_r fraction from Ultrogel AcA 44	32	0.066	2.06	47	3
Concentrated fraction bound to collagen–Sepharose	2.5*	0.041	16.4	29	24

* Protein concentration was estimated according to the semi-quantitative protein assay method of Moron–Lowry.

chromatography on collagen–Sepharose was further used because of its capacity to adsorb the enzyme selectively at low ionic strength: control experiments indicated that the plasma-membrane proteinase did not bind to plain Sepharose and immobilized proteins such as bovine serum albumin and casein; however, gelatin–Sepharose and collagen–Sepharose bound the enzyme efficiently (> 98%). The affinity-bound DPP IV was therefore eluted with 0.154 M-NaCl in buffer. Subsequent elution of the columns with either 1 M-NaCl or 4 M-urea did not elute any apparent residual bound protein. The DPP IV peak of M_r 100000 from Ultrogel AcA 44 was therefore dialysed free of NaCl and passed over a column of collagen–Sepharose. Bound material eluted with 0.154 M-NaCl possessed DPP IV activity (the specific activity from different preparations ranged from 6 to 33 μmol/4 h per mg). SDS/polyacrylamide-gel-electrophoretic analysis of this fraction under non-reducing conditions (Fig. 3, lane a) revealed a major 55000-M_r protein and several minor contaminating proteins. After incubation of this fraction with [^{3}H]Pr$^i_2$-*P*-F (previously shown to be a potent inhibitor of DPP IV), followed by SDS/polyacrylamide-gel electrophoresis and fluorography, the 55000-M_r silver-stained band co-migrated with a ^{3}H-labelled band under both non-reducing (Fig. 3, lane b) and reducing conditions (results not shown). Taken together, these data indicated that DPP IV is a collagen-binding protein consisting of two non-covalently linked 55000-M_r polypeptidic subunits.

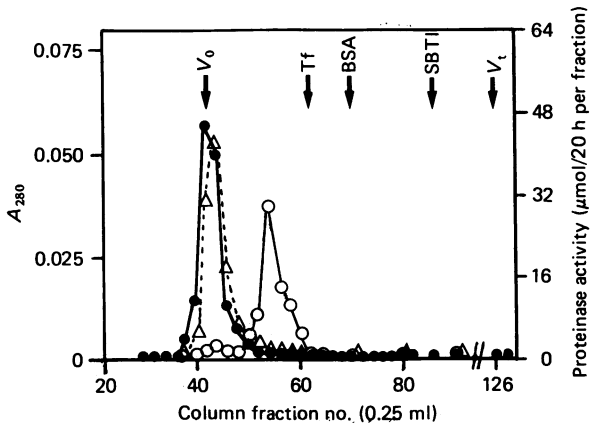

Fig. 2. Ultrogel AcA 44 chromatography of solubilized 3T12-cell plasma membranes

Solubilized plasma membranes (1 ml, 1 mg/ml) were applied to a column (0.5 cm × 35 cm) of Ultrogel AcA 44 equilibrated in 10 mM-citrate buffer, pH 6.0, containing 1 M-NaCl. Fractions (0.25 ml) were collected and analysed for protein content (●), for Gly-Pro-NH-Np hydrolysis (○) and for Leu-NH-Np hydrolysis (△). Arrows indicate the void volume (V_0) of the column determined by Dextran Blue 2000 and the elution volumes of transferrin (Tf, M_r 80000), bovine serum albumin (BSA, M_r 66000) and soya-bean trypsin inhibitor (SBTI, M_r 21500).

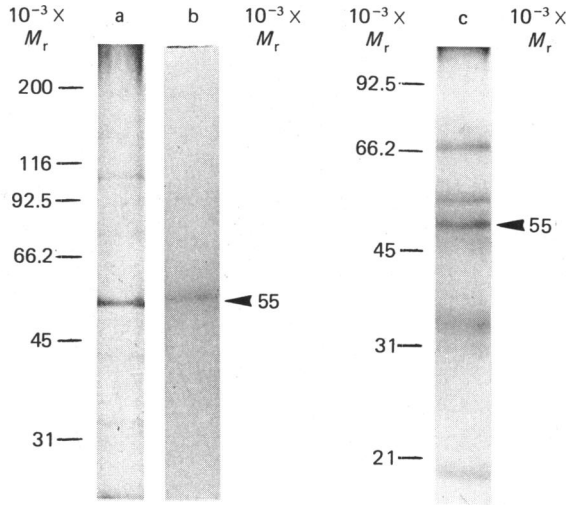

Fig. 3. SDS/polyacrylamide-gel electrophoresis of DPP-IV-active membrane samples

Lane a: concentrated DPP IV-active material from concentrated 100000-M_r Ultrogel fraction bound to collagen–Sepharose; electrophoresis was performed in a 7–12% polyacrylamide gel under non-reducing conditions, and proteins were stained with $AgNO_3$. Lane b: fluorography of the electrophoresed ^{3}H-labelled sample identical with lane a; the dried gel was exposed on to film for 8 weeks at −70 °C. Lane c: autoradiography of ^{125}I-labelled solubilized membrane material bound to collagen–Sepharose; electrophoresis was performed in a 10% polyacrylamide gel under non-reducing conditions, and the gel was exposed on to film for 3 weeks at −70 °C. M_r values of marker proteins are shown to the left of lanes a and c.

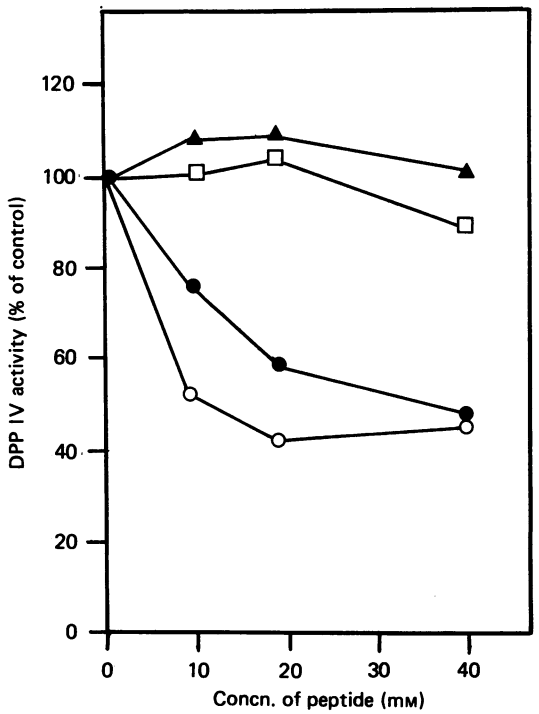

Fig. 4. Effect of di- and tri-peptide concentration of DPP IV activity from solubilized 3T12-cell plasma membranes

Solubilized plasma membranes were incubated in citrate buffer, pH 6.0, with 0.5 mM-Gly-Pro-NH-Np for 4 h and at 37 °C and in the absence (control) or in the presence of various concentrations of di- and tri-peptides: ●, Gly-Pro-Ala; ○, Ala-Pro-Gly; ▲, Gly-Pro; □, Gly-Gly-Gly.

DPP IV activity was completely retained on the lectin affinity-chromatography column of concanavalin–Sepharose, which specifically binds α-D-mannopyranosyl and α-D-glucopyranosyl molecules. A further attempt to purify DPP IV was therefore made by adsorbing the peptidase on the lectin–Sepharose. This did not lead to the elimination of a significant amount of contaminating proteins and recovery of DPP IV activity was significantly decreased (50–90 %).

After ^{125}I-radiolabelling of the intact fibroblasts, supernatants of detergent-solubilized whole ^{125}I-labelled cells were applied to collagen–Sepharose as in the purification of membrane DPP IV. As shown in Fig. 3 (lane c), autoradiography of the radiolabelled proteins contained in the collagen–Sepharose-bound fractions revealed a ^{125}I-labelled protein with an M_r of 55000 that did not change in reducing conditions (results not shown). In conclusion, these results suggest that the plasma-membrane-bound glycoprotein of M_r 55000 identified as DPP IV is exposed on the extracellular surface of the fibroblasts.

Effects of di- and tri-peptides on DPP IV activity and on 3T12 cell adhesion

Apparent inhibition of 3T12 membrane DPP IV activity by soluble peptides is shown in Fig. 4. The tripeptides Gly-Pro-Ala and Ala-Pro-Gly were found to be efficient inhibitors of the enzyme activity (approx. 50 % of control at 40 mM). In contrast, Gly-Gly-Gly and Gly-Pro had no inhibitory effect on DPP IV activity when tested in the same range of concentration. Furthermore, the specific ability of the tripeptides Gly-Pro-Ala and Ala-Pro-Gly to inhibit DPP IV relative to the other peptides was also observed (i) at neutral pH and (ii) with either 3T12-cell or 3T3-cell membrane preparations (results not shown). We further examined the effect of these various peptides on the adhesion of 3T12 cells to collagens and to fibronectin substrata. As shown in Table 3, the presence of 40 mM-Ala-Pro-Gly or -Gly-Pro-Ala inhibited collagen-mediated cell adhesion by nearly 40 % or more. In comparison with fibronectin-mediated cell adhesion, Ala-Pro-Gly was found to give only a weak inhibition (not more than 13 %). However, Gly-Pro-Ala showed a significant inhibition (60 %). The same result was obtained when fibronectin was coated directly on the micro-well (results not shown). Finally, neither of the two other reagents Gly-Gly-Gly and Gly-Pro had any significant effect on cell adhesion to collagens or to fibronectin (inhibition not more than 19 %).

Effect of partially purified DPP IV on 3T12 cell adhesion

After molecular exclusion chromatography and collagen affinity chromatography, a detergent-solubilized

Table 3. Effects of di- and tri-peptides on fibroblast adhesion

Adhesion of fibroblasts was measured in the absence or in the presence of peptides for 10 min on denatured and native collagens or for 5 min on fibronectin, at 37 °C with 5 mM-Mn^{2+} in the adhesion medium. Adhesion is expressed as the percentage of control attachment (with no addition). Results are the means for three experiments.

		Adhesion [% of control (no addition)]		
Inhibitor	Concn. (mM)	Denatured collagen	Native collagen	Fibronectin
Gly-Pro	20	102	123	96
	40	81	84	93
Gly-Gly-Gly	20	81	106	92
	40	83	90	89
Ala-Pro-Gly	20	76	90	90
	40	61	57	87
Gly-Pro-Ala	20	49	52	92
	40	33	50	40

DPP IV fraction was isolated and tested for blocking 3T12 cell adhesion. The detergent concentration used was 0.001%, a level that does not affect the appearance or viability of the cells or the stability of the insolubilized substrata. In two sets of experiments, the two different DPP IV preparations (specific activity values of 6 and 33 μmol/4 h per mg) presented cell-adhesion-disrupting activity on denatured collagen (30% inhibition) and on native collagen (18% and 55% inhibition with the two enzyme preparations). They were, however, without effect on fibronectin-mediated adhesion (not more than 5% inhibition).

DISCUSSION

By using affinity chromatography on a type I collagen–Sepharose I have isolated a glycoprotein from mouse fibroblast plasma membranes. Moreover, this protein appears to possess the ability to release specifically the dipeptide Gly-Pro from its *p*-nitroanilide. In view of its lack of activity with other synthetic substrates, the enzyme can be considered to be a dipeptidyl aminopeptidase IV (DPP IV). Type I collagen contains a high proportion of repeating Gly-Pro-Xaa triplets. Some are located in non-helical peptide chains (Becker *et al.*, 1975), whereas some others such as Gly-Pro-Met and Gly-Pro-Arg are present as the first and the third triplets in the helical region of both α1 and α2 chains (Fietzek & Kuhn, 1976). Thus fibroblast membrane DPP IV may be seen as possessing the potential for binding collagen or gelatin via accessible Gly-Pro sequences. To my knowledge, this is the first report that DPP IV can be purified by collagen affinity chromatography. However, the unusual sensitivity of the interaction between DPP IV and collagen at relatively low ionic strength may raise questions about the role of this interaction *in vivo*. The present result is consistent with those reported by Mollenhauer & Von der Mark (1983) and Dedhar *et al.* (1987), who found that collagen–Sepharose bound different collagen-binding proteins below physiological ionic strength. In both cases these proteins were dissociated from the collagen matrices at an ionic strength of 0.05–0.1 M-NaCl. However, when inserted into liposomes, those proteins bound collagen at salt concentrations of up to 0.5 M-NaCl. In consequence, Dedhar *et al.* (1987) suggested that the collagen-binding proteins, when associated with lipids, present an increase in affinity for collagen. Such observations suggest further study to clarify whether fibroblast membrane DPP IV interaction with collagen involves a 'lipid-associated peptidase' type.

Plasma-membrane localization of this enzyme has already been reported in BHK (baby-hamster kidney) fibroblasts and rat hepatocytes (Hanski *et al.*, 1984; Hixson *et al.*, 1984). DPP IV also appeared to be distributed in the extracellular connective-tissue matrices isolated from rat liver (Hixson *et al.*, 1984). In the present study, I did not consider the possibility that the biomatrix produced by the cultured mouse fibroblasts may have DPP IV activity. Further immunoprecipitation analysis with antibodies against purified DPP IV would provide an answer to this possibility.

Although the enzyme has optimal activity in the pH range 5–6, more than 50% of the optimal activity is still exhibited at neutral pH. DPP IV is not inhibited by the metal-ion chelator EDTA and by carboxy-group inhibitors. By contrast, Pr^{i}_{2}-*P*-F and phenylmethanesulphonyl fluoride abolish the activity of DPP IV, indicating that the fibroblast enzyme may therefore be considered to be a serine proteinase. From the results obtained by molecular exclusion chromatography on Ultrogel AcA 44 and SDS/polyacrylamide-gel electrophoresis (under non-reducing and reducing conditions), DPP IV appears to have an apparent M_r of 100000 represented by two non-covalently linked polypeptides of M_r 55000. In contrast, dipeptidyl aminopeptidases IV from other sources have been shown to be represented by two covalently linked subunits of M_r about 110000 (Kato *et al.*, 1978; Kreisel *et al.*, 1982).

Rubin *et al.* (1981) presented evidence that rat hepatocytes may attach to synthetic peptides with the collagen-like structures $(Gly\text{-}Pro\text{-}Pro)_n$, $(Gly\text{-}Pro\text{-}Hyp)_n$ and $(Gly\text{-}Ala\text{-}Pro)_n$, whereas no attachment was observed to polyproline (Rubin *et al.*, 1981). The cited authors suggested that the cell collagen-binding sites recognize a simple structure in collagen. On the other hand, Hanski *et al.* (1985) showed that the tripeptide Gly-Pro-Ala, as well as an antibody raised against mouse liver DPP IV, delayed fibronectin-mediated adhesion of rat hepatocytes on denatured collagen. Because DPP IV activity is present on the hepatocyte plasma membrane and can be inhibited by Gly-Pro-Ala (Hanski *et al.*, 1984), it was concluded that DPP IV may be involved in the initial phase of fibronectin-mediated cell spreading on collagen. Finally, the spreading of BHK cells (which too possess a DPP IV on their surface) was specifically triggered by Gly-Pro-Ala sequences covalently linked to an inert matrix consisting of bovine serum albumin. This process was exclusively inhibited by peptides affecting DPP IV activity (Hanski *et al.*, 1985). We previously showed that 3T3 and 3T12 fibroblasts possess the capacity to attach to collagens via a fibronectin-independent mediated mechanism (Bauvois & Roth, 1987). The present experiments in which fibroblast adhesion to collagens is inhibited by the two tripeptides Gly-Pro-Ala and Ala-Pro-Gly, which also inhibit fibroblast DPP IV activity, suggest the involvement of this enzyme in the interaction of cells with Gly-Pro sequences present in the collagen of the matrix. To address this more directly, I decided to purify the cell-surface DPP IV in order to generate the appropriate antiserum for examining the function of this enzyme in cell–matrix interactions. The preliminary purification procedure involving gel filtration and collagen affinity chromatography led to approx. 24-fold enrichment of the peptidase with a yield of 29% with respect to detergent-solubilized membranes. The DPP-IV-enriched fraction was able to disrupt cell–collagen interactions of 3T12 fibroblasts when added to the adhesion medium. It has to be noted that the tripeptide Gly-Pro-Ala strongly affected fibroblast adhesion to fibronectin adsorbed on denatured collagen. This inhibitory activity is surprising, but several explanations are plausible. Since fibronectin contains a specific domain binding to collagen, one likely hypothesis is that Gly-Pro-Ala–fibronectin interaction results in conformational alterations within the fibronectin molecule, which inhibits recognition of the fibroblast fibronectin receptor. Alternatively, the tripeptide may alter the cell surface by binding to membrane components (different from DPP IV). Further study is needed to provide an answer to this problem.

The involvement of cell-surface enzyme activities other

than peptidase activity and serving as the basis for adhesion phenomena between cells and substrata has already been reported. For example, Rauvala & Hakomori (1981) described an α-mannosidase on the surface of hamster-embryo fibroblasts that mediates cell adhesion based on the recognition of high-mannose-type glycoproteins in the matrix. As initially suggested by Roseman (1970), glycosyltransferases may have similar properties. Shur (1983) suggested that a surface galactosyltransferase of embryonal carcinoma cells participate in cell adhesion by recognizing lactosaminoglycans on adjacent cell surfaces. We demonstrated previously that mouse fibroblasts possess at their surface glucosyltransferases recognizing the galactosyl-hydroxylysyl residues on collagenous proteins (Bauvois & Roth, 1985). However, pretreatment of collagen either with β-galactosidase or by oxidation of terminal galactosyl residues, which inhibited glucosyltransferase activity, did not modify fibroblast adhesion to collagen (B. Bauvois, unpublished work). Thus the collagen–glucosyltransferase interaction does not appear to be involved in the initial attachment of cells to collagen.

In summary, the results of the present study demonstrate the localization of a DPP IV at the surface of mouse fibroblasts. Preliminary evidence for the involvement of this enzyme in cell–collagen interaction is obtained from (i) the specific ability of tripeptides to inhibit the DPP IV activity and adhesion on collagen and (ii) disruption of cell adhesion to collagens by a DPP-IV-enriched preparation. Whether the DPP IV serves as anchorage site for collagen on the fibroblast surface *in situ* remains to be resolved, however. Variants defective in both peptidase activity and attachment to collagen matrices would be valuable tools to determine the biological function(s) of this enzyme.

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