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Evidence for a role of dipeptidyl peptidase IV in fibronectin-mediated interactions of hepatocytes with extracellular matrix

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Dipeptidyl peptidase IV (DPP IV) is a cell surface glycoprotein which has been implicated in hepatocyte–extracellular matrix interactions [Hixson, DeLourdes, Ponce, Allison & Walborg (1984) Exp. Cell Res. **152**, 402–414; Walborg, Tsuchida, Weeden, Thomas, Barrick, McEntire, Allison & Hixson (1985) Exp. Cell Res. **158**, 509–518; Hanski, Huhle & Reutter (1985) Biol. Chem. Hoppe-Seyler **366**, 1169–1176]. However, its proteolytic subtrate(s) and/or binding protein(s) which mediate this influence have not been conclusively identified. Nitrocellulose binding assays using ^{125}I-labelled DPP IV that was purified to homogeneity from rat hepatocytes revealed a direct interaction of DPP IV with fibronectin. Although fibronectin could mediate an indirect binding of DPP IV to collagen, no evidence was found for a direct binding of DPP IV to native or denatured Type I collagen. Fibronectin appeared to bind DPP IV at a site distinct from its exopeptidase substrate recognition site since protease inhibitors such as competitive peptide substrates and phenylmethanesulphonyl fluoride enhanced binding, possibly as a result of an altered conformation of DPP IV. To determine if fibronectin binding to DPP IV is involved in the interaction of fibronectin with the hepatocyte surface, the effect of various DPP IV inhibitors on ^{125}I-fibronectin binding to isolated hepatocytes in suspension was examined. Kinetic studies revealed that inhibitors of DPP IV which enhanced fibronectin binding *in vitro* accelerated the initial binding of fibronectin to the cell surface where it was subsequently cross-linked (presumably by tissue transglutaminase) to as yet undefined components. Immunolocalization of fibronectin and DPP IV in normal rat liver sections showed that both proteins were present along the hepatocyte sinusoidal membrane. These observations, coupled with previous results showing that DPP IV is tightly bound to biomatrix isolated from rat liver (Hixson *et al.*, 1984; Walborg *et al.*, 1985), suggest that DPP IV binding to fibronectin may play a role in interactions of hepatocytes with extracellular matrix *in vivo* and possibly in matrix assembly.

INTRODUCTION

Dipeptidyl peptidase IV (DPP IV) is a serine exoprotease that was first identified in rat kidney by its ability to cleave X-proline or X-alanine dipeptides from the *N*-terminus of certain peptides (Hopsu-Havu & Glenner, 1966). Subsequent studies have shown that DPP IV exists in a number of tissues, including liver where high concentrations are present in bile canalicular membrane domains (Hixson *et al.*, 1984; Walborg *et al.*, 1985). DPP IV is a sialoglycoprotein (Fukasawa *et al.*, 1978) that is anchored to the plasma membrane through a hydrophobic *N*-terminal domain such that most of its molecular mass is exposed to the outside of the cell (Macnair & Kenny, 1979; Hong & Doyle, 1988). Under non-denaturing conditions, DPP IV exists as a dimer having disulphide-linked subunits with an apparent M_r value of 150000 (105000 after denaturation).

Previous evidence suggests an involvement of DPP IV in hepatocyte–extracellular matrix interactions. Hixson *et al.* (1984), using antiserum against hepatocyte membranes, showed that a 105000-M_r cell surface polypeptide, designated Hep 105, was tightly bound to isolated cell-free preparations of liver biomatrix. Subsequent studies positively identified Hep 105 as a denatured polypeptide derived from the 150000-M_r monomer of DPP IV (Walborg *et al.*, 1985). More recently it has been demonstrated that competitive substrates of DPP IV decrease the rate of hepatocyte (Hanski *et al.*, 1985, 1988) and fibroblast (Bauvois, 1988) spreading on matrices of fibronectin and/or collagen. As yet, the proteolytic substrate and/or binding sites of DPP IV which mediate this influence on cell adhesion have not been conclusively identified. Because of the unusual specificity of DPP IV for proline-containing residues, it has been proposed that DPP IV cleaves collagen, a proline-rich polypeptide (Hopsu-Havu & Ekfors, 1969) although there has been no direct evidence indicating that collagen or collagen-derived peptides are degraded by DPP IV. DPP IV has been shown to bind immobilized collagen (Hanski *et al.*, 1985; Bauvois, 1988). However, this putative interaction did not involve the catalytic site of DPP IV and may have been mediated by fibronectin as we report here.

Fibronectin has been proposed to interact with cells through a number of different mechanisms. One process involves a complex of membrane glycoproteins belonging to the integrin family which have affinity for Arg-Gly-Asp residues on the cell binding domain of fibronectin (Ruoslahti & Pierschbachser, 1987). Fibro-

Abbreviations used: DPP IV, dipeptidyl peptidase IV; AFC, amino-4-trifluoromethylcoumarin; MNA, 4-methoxy-β-naphthylamide; PAGE, polyacrylamide-gel electrophoresis; t.i.u. trypsin inhibitory units; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; Z-, benzyloxycarbonyl; WGA, wheat-germ agglutinin; MAb, monoclonal antibody; PMSF, phenylmethanesulphonyl fluoride; NP-40, Nonidet P-40.

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nectin can also interact with the cell surface through a site localized near its *N*-terminus that has been implicated in matrix assembly (McDonald *et al.*, 1987). Although an integrin-like complex has been isolated from hepatocytes (Johansson *et al.*, 1987), recent evidence suggests that fibronectin can bind hepatocytes with high affinity independent of Arg-Gly-Asp residues (Hughes & Stamatoglou, 1987; Fellin *et al.*, 1988). Hence, non-integrin membrane receptors for fibronectin are likely to exist on hepatocyte surfaces and may play an important regulatory role in certain aspects of cell–extracellular matrix interactions such as matrix assembly.

In the present paper, evidence is presented from studies *in vitro* showing a novel interaction of purified rat hepatocyte DPP IV with fibronectin. Experiments measuring the binding of fibronectin to hepatocytes in suspension and immunolocalization studies suggest that this interaction contributes to interactions of hepatocytes with extracellular matrix *in vivo*.

EXPERIMENTAL

Materials

Wheat-germ agglutinin (WGA) immobilized to agarose (5 mg of lectin/ml of gel) was from Pharmacia LKB Biotechnology; immuno-peroxidase-conjugated second antibodies and diaminobenzidene were from Cappel; purified laminin from the mouse EHS tumour was from Collaborative Research; paraformaldehyde and glutaraldehyde were from Polysciences; benzoyl-carbonyl-Gly-Pro-4-methoxy-β-naphthylamide was from Serva; IgG Sorb used for immunoprecipitation was from The Enzyme Center; ^{125}I (NaI in NaOH) was from Amersham; cellulose acetate sheets impregnated with Ala-Pro-amino-4-trifluoromethylcoumarin (AFC) for enzyme overlay were from Enzyme Systems; polyacrylamide-gel reagents were from Bio-Rad and antiserum raised against purified rat plasma fibronectin was a gift from Dr. John Glenney (University of Kentucky, Lexington, KY, U.S.A.). All other chemicals and reagents were from Sigma Chemical Co.

Purification and assay of hepatocyte DPP IV

DPP IV was purified from hepatocytes isolated from adult male ACI rats (Harlan Industries, Indianapolis, IN, U.S.A.) by perfusion of the intact liver with collagenase as described previously (Hixson *et al.*, 1983). Non-ionic detergent extracts were made by lysing washed hepatocytes from one liver for 30 min on ice in lysis buffer (2 ml/ml of packed cells) containing 10 mM-Tris/HCl, pH 8.0/0.5% Nonidet P-40 (NP-40)/0.15 M-NaCl/0.04 trypsin inhibitory units (t.i.u.) of aprotinin/ml. Detergent-insoluble material was removed by centrifugation at 35000 *g* for 30 min at 4 °C. For binding studies, the detergent extract was subjected to affinity chromatography over immobilized WGA as described previously by Elovson (1980). After washing with lysis buffer, bound components were eluted with 0.5 M-*N*-acetylglucosamine dissolved in the same buffer and dialysed against 20 mM-Tris/HCl, pH 7.8, containing 0.1% Tween-20. Radiolabelled DPP IV was prepared by iodinating the WGA-bound fraction using the chloramine-T method (Romani *et al.*, 1986). DPP IV was purified to homogeneity by immunoprecipitating non-ionic detergent extracts or iodinated WGA-binding proteins with formalin-fixed *Staphylococcus aureus* (IgG Sorb) that had been previously coupled with monoclonal antibody (MAb) 236.3, specific for DPP IV (Walborg *et al.*, 1985). After washing three times with lysis buffer containing 0.1% deoxycholate, bound components were eluted with 1 ml of 3 M-potassium thiocyanate dissolved in 20 mM-Tris/HCl, pH 7.8, containing 0.1% Tween-20, and dialysed at 4 °C in this buffer without potassium thiocyanate.

DPP IV activity in solution was determined photometrically using the chromogenic substrate Gly-Pro-MNA. Reaction mixtures were prepared in volumes of 0.625 ml with 20 mM-Tris/HCl, pH 7.8. The reaction was initiated by the addition of 0.125 ml of 1 mM-Gly-Pro-MNA, allowed to proceed for 30 min at 37 °C, and stopped by the addition of 0.5 ml of 1 M-sodium acetate, pH 4.2, containing 1 mg of Fast Garnet GBC/ml and 0.1% Tween-20. Enzyme activity, as measured by the concentration of the leaving group, MNA, was determined by measuring absorbance of the reaction mixture at 525 nm. One unit of enzyme activity was defined as the amount of enzyme required to cleave 1 μmol of product/min at 37 °C and was determined by absorbance of known concentrations of MNA.

DPP IV was identified enzymically after separation by polyacrylamide-gel electrophoresis (PAGE) in the presence of 0.1% SDS by overlaying the unfixed gel for 15–30 min at 37 °C with a sheet of cellulose acetate impregnated with the fluorogenic substrate Ala-Pro-AFC (Smith, 1984).

Purification of plasma fibronectin

Fibronectin was purified from plasma drawn from normal human donors by successive affinity chromatography over gelatin–agarose and heparin–agarose as described (Hayashi & Yamada, 1983). Freshly isolated plasma was made to 5 mM-EDTA and 1 mM-phenylmethanesulphonyl fluoride (PMSF) to inhibit plasma proteases.

Nitrocellulose binding assay

A nitrocellulose binding assay based on previously described methods (Wallace *et al.*, 1987) was developed to detect DPP IV-binding proteins in crude hepatocyte extracts. SDS-solubilized proteins were separated by SDS/PAGE and transferred to nitrocellulose as described by Towbin *et al.* (1979). The protein blots were then incubated overnight at 4 °C with Tris-buffered saline (TBS; 10 mM-Tris/HCl, pH 7.4/150 mM-NaCl) containing 5% (w/v) non-fat dry milk to block potential non-specific binding. After washing three times in TBS (50 ml each for 30 min), the blots were incubated for 2 h at 4 °C with ^{125}I-labelled DPP IV (1×10^5 c.p.m./ml) diluted in TBS containing 0.05% NP-40 in the presence or absence of DPP IV inhibitors. Unbound ^{125}I-DPP IV was removed with three washes of TBS containing 0.05% NP-40 and the inhibitor. The blots were then air dried and autoradiographed to detect DPP IV-binding proteins.

DPP IV binding to fibronectin-coupled gelatin–agarose

Gelatin–agarose (Sigma) was mixed by rotation for 2 h at 4 °C with fetal-calf serum (1:5, w/v) as a source of fibronectin. Unbound material was removed by washing three times with binding buffer (25 mM-$NaHCO_3$, pH 7.4/150 mM-NaCl/5 mM-$CaCl_2$). Aliquots (0.5 ml) of fibronectin-coupled gelatin–agarose suspended in binding buffer (1:3, w/v) were rotated end-over-end with

purified ^{125}I-DPP IV (5×10^5 c.p.m./ml) for 1 h at 4 °C. Unbound ^{125}I-DPP IV was removed by centrifuging 0.5 ml aliquots at 10 000 *g* for 1 min through 0.5 ml of binding buffer containing 4% (w/v) Ficoll that was overlaid with 0.2 ml of binding buffer. After excising the tip of the tube, bound ^{125}I-DPP IV was quantified using a gamma counter.

Immunofluorescence detection of DPP IV binding to fibronectin-coupled collagen fibrils

DPP IV binding to native and fibronectin-coupled collagen fibrils was measured essentially as described by Hanski *et al.* (1988). Type I collagen that was purified from rat tail tendons using the procedure of Strom & Michalopoulos (1982) was dried overnight on plastic cell culture cover slips. After washing with phosphate-buffered saline (PBS; 140 mM-NaCl/4 mM-KCl/2 mM-KH_2PO_4/20 mM-Na_2HPO_4, pH 7.4), fibrils coated with fibronectin were incubated at room temperature for 30 min with affinity-purified fibronectin at a concentration of 0.1 mg/ml dissolved in 20 mM-Tris/HCl, pH 7.8, containing 150 mM-NaCl. After washing with 0.02 M-Tris/HCl, pH 7.8, containing 0.1% Tween-20, untreated or fibronectin-coated fibrils were incubated with purified DPP IV at room temperature for 30 min. After removing unbound DPP IV by washing three times, the coverslips were fixed in acetone for 10 min at 4 °C. Bound DPP IV was labelled with MAb 236.3 by indirect immunofluorescence and revealed by fluorescence microscopy as described below.

Binding of fibronectin to isolated rat hepatocytes in suspension

Fibronectin binding to isolated rat hepatocytes was measured essentially as described by Fellin *et al.* (1988). In brief, hepatocytes isolated by collagenase perfusion from normal rat liver were centrifuged through a Percoll gradient (Dalet *et al.*, 1982) to remove dead or damaged cells, thereby yielding a cell preparation with 95–99% viability. Hepatocytes were washed and suspended at 2.5×10^6 cells/ml in binding buffer containing 50 μg of leupeptin/ml, 0.04 t.i.u. of aprotinin/ml, 0.1 μg of pepstatin/ml and 0.05 units of hirudin/ml. Following the sequential addition of 0.1 vol. of a 10 × concentrated solution of various DPP IV inhibitors and an equal volume of 50 nM-^{125}I-fibronectin (Romani *et al.*, 1986), hepatocytes were rotated end-over-end for up to 2 h at 4 °C. At various times, 200 μl aliquots were removed, layered on to a cushion composed of 100 μl of binding buffer with protease inhibitors over 200 μl of this same buffer containing 4% (w/v) Ficoll, and centrifuged at 10000 *g* for 1 min. The tip of the tube containing the hepatocyte pellet was excised and counted in a gamma counter. The pellet was solubilized in 500 μl of SDS sample buffer, boiled for 2 min, and 100 μl aliquots were analysed by SDS/PAGE.

Immunohistochemistry

Immunofluorescence. Frozen sections of rat liver, 4–6 μm thick, were fixed in cold acetone for 10 min, labelled by indirect immunofluorescence with MAb 236.3 or anti-fibronectin antiserum (rabbit) followed by fluorescein-conjugated, affinity-purified, goat anti-(mouse IgG) or goat anti-(rabbit IgG) respectively, as previously described (Hixson *et al.*, 1983), and examined on a Nikon Microphot-FX fluorescence microscope.

Mechanically dissociated hepatocytes. After perfusion fixation of normal rat liver with ice-cold PBS containing 2% (w/v) paraformaldehyde and 0.1% glutaraldehyde, the liver was excised, cut into thin slices (1–2 mm) and placed in fresh fixative for 15 min. Liver slices were mechanically disrupted in PBS using a loose fitting Dounce homogenizer. To enrich for hepatocyte doublets, the suspension was filtered over six layers of gauze to remove large aggregates and centrifuged at 550 *g* for 1 min.

Immunoperoxidase. After washing in 0.1 M-glycine/PBS and 2% (v/v) normal goat serum, mechanically dissociated hepatocytes were incubated with primary antibody for 2 h at 4 °C, washed in PBS, and incubated in peroxidase-conjugated goat anti-(mouse IgG) for 2 h, followed by 0.05% diaminobenzidene and 0.01% H_2O_2 for 15 min. Samples were postfixed in 3% (v/v) glutaraldehyde in Millonig's PBS and osmium tetroxide, dehydrated in ethanol, and embedded in Spurr's resin using a standard protocol. Ultrathin sections were examined with a Philips 300 electron microscope.

Other methods

SDS/PAGE was performed using the buffer system described previously by Laemmli (1970).

RESULTS AND DISCUSSION

Purification and characterization of DPP IV from rat hepatocytes

DPP IV was purified from isolated rat hepatocytes by immunoprecipitation with Mab 236.3 that was previously shown to react specifically with DPP IV (Walborg *et al.*, 1985). Analysis by SDS/PAGE after heat denaturation showed that immunoprecipitates from iodinated WGA-binding proteins were homogeneous for a 105000-M_r polypeptide (Fig. 1, lane b). If the sample was not boiled, lesser amounts of the 105000-M_r polypeptide were evident, and a 150000-M_r polypeptide appeared (Fig. 1, lane c). Heat denaturation of gel slices containing the 150000-M_r polypeptide (results not shown) yielded silver-stained polypeptides with M_r values by SDS/PAGE of 105000 and 50000 (not detected when iodinated). This finding, and previous reports showing that the 150000- and 105000-M_r polypeptides generated similar peptide maps after limited protease digestion (Walborg *et al.*, 1985), indicate that the 105000-M_r polypeptide was derived from the 150000-M_r monomer of DPP IV.

In the unboiled sample, only the 150000-M_r polypeptide displayed dipeptidase activity as determined by its ability to cleave fluorogenic dipeptide substrates following electrophoretic separation (Fig. 1, lane d). Although no enzymic activity was associated with the 105000-M_r polypeptide, it has been previously demonstrated that this fragment of DPP IV contains the catalytic site since it could be labelled with [^{3}H]diisopropyl fluorophosphate (Walborg *et al.*, 1985). Preparations of immunoprecipitated DPP IV were generally composed of at least 50% of the enzymically inactive 105000-M_r form of DPP IV.

The DPP IV preparation was further characterized by assay using the chromogenic substrate Gly-Pro-MNA, in the presence of various protease inhibitors. As shown in Table 1, dipeptidase activity was heat sensitive and effectively blocked by irreversible sulphonylation of the

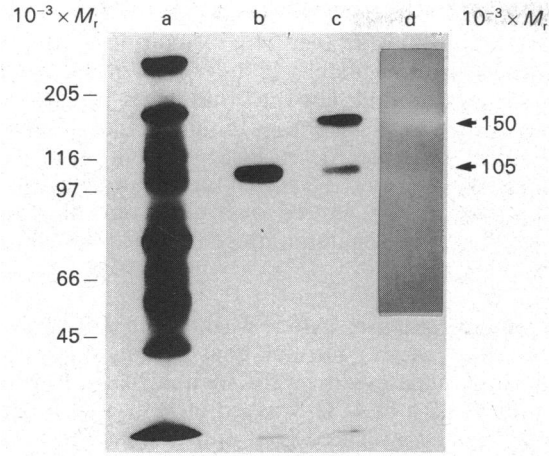

Fig 1. SDS/PAGE of ^{125}I-DPP IV purified from rat hepatocytes

^{125}I-DPP IV purified as described under the Experimental section was subjected to SDS/PAGE under reducing conditions on gels of 7.5% polyacrylamide, and revealed by either autoradiography (lanes a–c) or enzyme overlay (lane d). Lane a (2 h exposure) shows composition of ^{125}I-labelled WGA-binding protein fraction from which DPP IV was purified. Lanes b and c (48 h exposure) are of identical aliquots of purified ^{125}I-DPP IV that were either boiled (b), or incubated at room temperature (c) for 5 min in SDS sample buffer prior to SDS/PAGE. Lane d is an enzyme overlay of lane c using cellulose acetate impregnated with the fluorogenic substrate Ala-Pro-AFC.

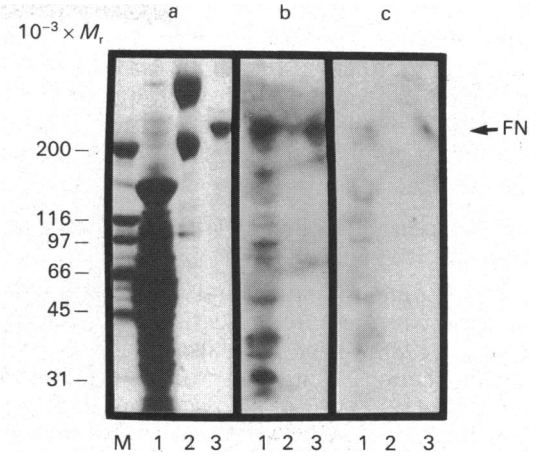

Fig. 2. Identification of fibronectin as a major DPP IV-binding protein in whole hepatocyte extracts

Whole hepatocyte detergent extract (200 μg) (lanes 1), purified laminin (50 μg) (lanes 2) and purified fibronectin (20 μg) (lanes 3) were subjected to SDS/PAGE under reducing conditions on gradients of 5–15% polyacrylamide. Following electrophoresis, the samples were either stained with Coomassie Brilliant Blue (panel a), or transferred to nitrocellulose and incubated with purified ^{125}I-DPP IV (see Fig. 1 for purity) in the presence (panel b) or absence (panel c) of 1 mM-Gly-Pro-Ala as described in the Experimental section. The position of fibronectin (FN) is shown to the right of the Figure by an arrow. Lane M shows molecular mass markers. Autoradiographs in panels b and c were both exposed for 7 days.

Table 1. Sensitivity of the dipeptidase activity of purified DPP IV to various protease inhibitors

DPP IV was purified by immunoprecipitating non-ionic detergent extracts of rat hepatocytes with MAb 236.3 and assayed for exopeptidase activity using the chromogenic substrate Gly-Pro-MNA as described in the Experimental section. A 10 x concentrated stock solution of each inhibitor was added to the reaction mixture just before initiating the assay by the addition of DPP IV. Values of enzyme activity are the means of duplicate samples.

Volume assayed (μl)	Treatment	Dipeptidase activity (munits)	Dipeptidase activity (% of control)
100	None (control)	1.61	100.0
25	None	0.36	22.3
50	None	0.98	60.9
100	Heat inactivation, (100 °C, 10 min)	0.01	0.6
100	1 mM-PMSF	0.00	0.0
100	0.04 t.i.u. of aprotinin/ml	1.32	82.0
100	20 mM-Gly-Pro-Ala	0.16	9.9
100	10 mM-Val-Pro-Leu	0.03	1.9

active-site serine residue by PMSF. Aprotinin, which is selective for trypsin-like endoproteases, was not effective. The synthetic tripeptides Gly-Pro-Ala and Val-Pro-Leu also inhibited dipeptidase activity, presumably by competing with the chromogenic substrate for the substrate recognition site and/or catalytic site. The possibility that the immunoprecipitated enzyme was a proline-specific endopeptidase was ruled out because it was unable to cleave the *N*-terminal-blocked chromogenic substrate Z-Gly-Pro-MNA. These results indicated that the immunoprecipitated material displayed inhibitor sensitivity characteristic of DPP IV.

Identification of fibronectin as a DPP IV-binding protein in whole hepatocyte extracts

A nitrocellulose binding assay was developed for the purpose of identifying novel proteins from crude cell extracts which interact with DPP IV. Similar techniques have been employed to identify other protein–protein interactions (Wallace *et al.*, 1987) and depend on the ability of the binding site to retain its properties after SDS denaturation. As shown in Fig. 2, the presence of the competitive tripeptide substrate, Gly-Pro-Ala, unexpectedly enhanced DPP IV binding to several polypeptides from SDS-solubilized whole hepatocyte extracts, including two predominant polypeptides with M_r values of 220000 and 30000 (lane 1, panel b). DPP IV binding was either lost (30000-M_r polypeptide) or greatly reduced (220000-M_r and other binding proteins) when the dots were incubated with DPP IV in the absence of Gly-Pro-Ala (lane 1, panel c). Similar results were obtained if the blots were incubated with DPP IV in the presence of other DPP IV inhibitors such as PMSF (results not shown). The ability of DPP IV inhibitors to enhance DPP IV binding to specific hepatocyte proteins suggests that these interactions are the result of a direct binding to

Table 2. ^{125}I-DPP IV binding to fibronectin–gelatin–agarose in the presence of DPP IV inhibitors

See the Experimental section for details of the experiment. Values shown are the means of duplicate samples.

Affinity resin	Treatment	Bound DPP IV (c.p.m.)	Bound DPP IV (% of control)
Agarose (control)	None	2645	100
Gelatin–agarose	None	2574	97
Fibronectin–gelatin–agarose	None	4077	154
Fibronectin–gelatin–agarose	1 mM-PMSF	6404	242
Fibronectin–gelatin–agarose	20 mM-Gly-Pro-Ala	5149	194
Fibronectin–gelatin–agarose	10 mM-Val-Pro-Ala	5942	225

DPP IV and do not involve a contaminating polypeptide. In addition, these results, as well as those described below, suggest that the binding site on DPP IV for these proteins is distinct from the substrate recognition and/or catalytic site(s). Although the mechanism by which DPP IV inhibitors enhance binding is not known, it is conceivable that such inhibitors induce an enzyme conformation which favours protein binding.

The predominant 220000-M_r DPP IV-binding protein from whole hepatocyte extracts was identified as fibronectin by the ability of purified plasma fibronectin to bind DPP IV under identical conditions (lane 3, panels b and c). Plasma fibronectin displayed a mobility after SDS/PAGE run under reducing conditions identical to the 220000-M_r DPP IV-binding protein from whole hepatocyte extracts (lane 3, panels a and b). In addition, the 220000-M_r DPP IV-binding protein cross-reacted with anti-fibronectin antiserum as determined by immunoblots (results not shown). Laminin, which is functionally related to fibronectin, but structurally distinct, did not bind DPP IV under identical conditions even though 2.5 times more protein was loaded on the gel (lane 2, panels b and c). The lower-M_r range DPP IV-binding proteins present in whole hepatocyte extracts have yet to be identified, and the possibility that some may represent proteolytic fragments of fibronectin has not been ruled out.

Effect of fibronectin on the binding of DPP IV to collagen

DPP IV binding to fibronectin was further investigated by reacting purified ^{125}-DPP IV with plasma fibronectin that had been coupled to gelatin–agarose. The results of one such experiment are shown in Table 2. Contrary to previous reports suggesting a direct interaction of DPP IV with gelatin-affinity matrices (Bauvois, 1988; Hanski *et al.*, 1988), we observed no binding of DPP IV to gelatin–agarose (see below). Fibronectin-coupled gelatin–agarose displayed a modest increase (1.5-fold) in DPP IV binding relative to the agarose and bovine serum albumin agarose (results not shown) controls. In the presence of competitive tripeptide substrates of DPP IV, binding to fibronectin-coupled gelatin–agarose was increased to a level 2.0–2.3-fold greater than that observed with the agarose control. PMSF also enhanced DPP IV binding to fibronectin-coupled gelatin–agarose by 2.4-fold over the agarose control. Similar results were obtained if fibronectin was coupled to heparin–agarose and reacted with ^{125}I-DPP IV (results not shown). These results, in agreement with those obtained from the nitrocellulose binding assay, show low-level, but reproducible and detectable binding of DPP IV to fibronectin in the absence of DPP IV inhibitors. The enhancement effect of DPP IV-specific substrates and active-site-directed inhibitors suggest, as discussed above, that this interaction is specific and occurs at a site distinct from the exopeptidase catalytic domain of DPP IV.

While these studies were in progress, several reports appeared which suggested that DPP IV directly bound immobilized collagen affinity matrices (Bauvois, 1988; Hanski *et al.*, 1988). However, in both studies, binding was only apparent using buffers with salt concentrations that were not physiological and were 10-fold lower than those used in the binding experiments described above. This suggests that the affinity of DPP IV for fibronectin is much higher than its reported affinity for collagen. Indeed, careful analysis of binding results reported by Hanski *et al.* (1988) would favour this possibility. To further investigate the putative interaction of DPP IV with collagen, we used an identical source of collagen and similar protocol and conditions to those used in the study of Hanski *et al.* (1988). Untreated collagen fibrils that were incubated with purified DPP IV did not bind DPP IV-specific MAb 236.3 as shown in Fig. 3(*a*). However, if the fibrils were coated with fibronectin prior to incubating with purified DPP IV, the fibrils were labelled with the DPP IV MAb (Fig. 3*b*). The presence of Gly-Pro-Ala in the incubation buffer with purified

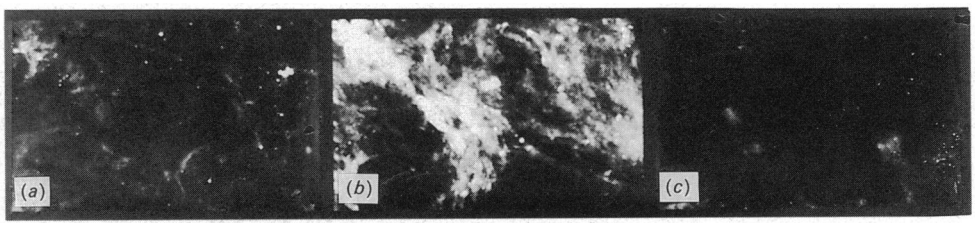

Fig. 3. Effect of fibronectin on the binding of DPP IV to Type I collagen

Native Type I collagen fibrils (*a*) or fibronectin-coupled Type I collagen fibrils (*b* and *c*) were incubated with purified DPP IV and labelled with MAb 236.3 against DPP IV (*a* and *b*) or P3X63Ag8 mouse myeloma culture supernatant (*c*) as described in the Experimental section. Micrographs were photographed and printed at identical exposure times to display intensities. Magnification × 142.

DPP IV resulted in more intense labelling to fibronectin-coupled collagen fibrils, but had no apparent effect on untreated fibrils (results not shown). These observations suggest that in the studies of Bauvois (1988) and Hanski *et al.* (1988), DPP IV binding to collagen resulted from an indirect interaction mediated by fibronectin that possibly contaminated solubilized plasma membrane preparations that were used as a source of DPP IV.

Effect of DPP IV inhibitors on ^{125}I-fibronectin binding to hepatocytes in suspension

To determine if binding of fibronectin to DPP IV plays a role in the interaction of fibronectin with the hepatocyte surface, the effects of various DPP IV inhibitors on the binding of ^{125}I-fibronectin to isolated hepatocytes in suspension were measured. As recently characterized by Fellin *et al.* (1988), fibronectin binds in a time-dependent manner to hepatocytes maintained in suspension at 4 °C. Under conditions employed by these investigators, fibronectin binding was not inhibited by Arg-Gly-Asp-containing peptides, suggesting that an integrin-like molecule did not play a role in this interaction. As shown in Fig. 4(*a*), PMSF and competitive tripeptide substrates of DPP IV accelerated the binding of fibronectin to hepatocytes. The stimulatory effect of the inhibitors was most apparent during the initial phase of binding to hepatocytes. For example, fibronectin binding in the presence of Val-Pro-Leu resulted in an approx. 2-fold increase from the control condition after incubating for 30 min. These findings are consistent with results from binding studies *in vitro* using purified DPP IV as described above, and observations that the catalytic site of DPP IV is exposed on the cell surface (G. A. Piazza, H. M. Callanan, J. Mowery & D. C. Hixson, unpublished work). The fact that the stimulatory effect of DPP IV inhibitors was observed using nanomolar concentrations of fibronectin provided indirect evidence that this interaction is of relatively high affinity. Our finding may thus relate directly to the cell surface receptor proposed by McKeown-Longo & Mosher (1985) that binds fibronectin with high affinity relative to integrin and functions in matrix assembly.

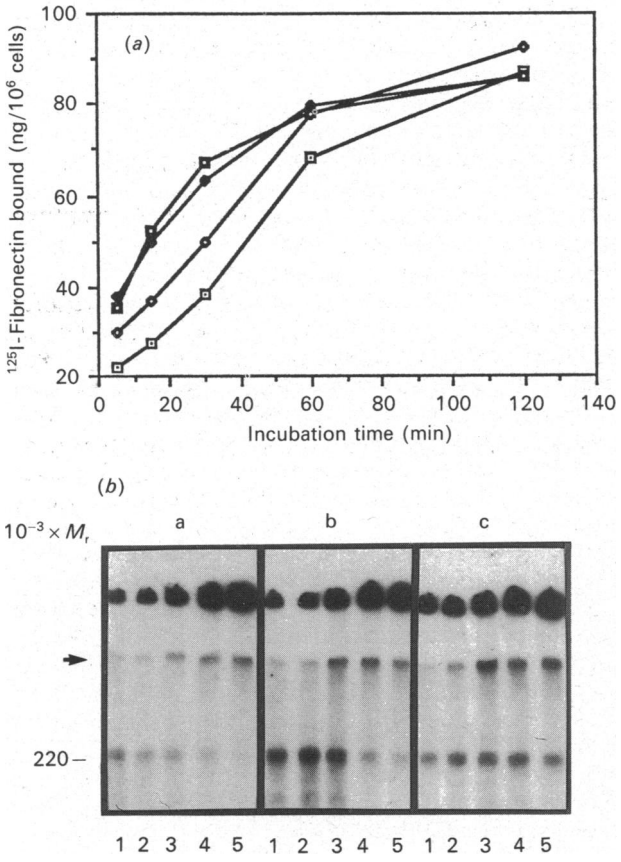

Fig. 4. Effect of DPP IV inhibitors on ^{125}I-fibronectin binding to hepatocytes in suspension

(*a*) Time course of ^{125}I-fibronectin binding to rat hepatocytes in the presence of 20 mM-Gly-Pro-Ala (◇), 10 mM-Val-Pro-Leu (◆), 1 mM-PMSF (■) or no inhibitor (□). Values shown are the means of duplicate samples. (*b*) SDS/PAGE of hepatocyte pellets after incubating with ^{125}I-fibronectin without inhibitor (panel a), or in the presence of 1 mM-PMSF (panel b) or 10 mM-Val-Pro-Leu (panel c). Hepatocyte pellets were solubilized in SDS sample buffer and boiled for 5 min prior to SDS/PAGE under reducing conditions in gels of 5% polyacrylamide. Lanes 1–5 show 5, 15, 30, 60, and 120 min incubations with ^{125}I-fibronectin respectively. The arrow indicates where the stacking gel meets the resolving gel. See the Experimental section for further details.

To determine how fibronectin was processed during the course of the reaction, aliquots containing equivalent numbers of hepatocytes from binding experiments performed in the presence or absence of DPP IV inhibitors (Fig. 4*a*) were solubilized in SDS sample buffer and separated by SDS/PAGE under reducing conditions. As shown in Fig. 4(*b*), the initial binding of fibronectin (shown by the 220000-M_r monomer) to the hepatocyte surface was more significantly enhanced by DPP IV inhibitors such as Val-Pro-Leu and PMSF than indicated from measures of total radioactivity (Fig. 4*a*). Densitometry of the 220000-M_r monomer fibronectin band revealed that Val-Pro-Leu enhanced binding by 3–4-fold and PMSF enhanced binding by 8-fold over binding in the absence of inhibitors after a 30 min incubation. In agreement with the report of Fellin *et al.* (1988), during the later time periods of the reaction fibronectin was covalently cross-linked into a high-M_r complex that did not enter the resolving gel. As shown previously, the high-M_r complex was composed of fibronectin cross-linked to other undefined component(s) (Fellin *et al.*, 1988). Its formation is presumably mediated by tissue transglutaminase since it is calcium-dependent and blocked by various transglutaminase inhibitors and substrates (Fellin *et al.*, 1988). Moreover, studies by Tyrrell *et al.* (1988) have recently demonstrated that fibronectin is a major component of a high-M_r transglutaminase substrate isolated from rat liver. These reports, together with studies showing that DPP IV is tightly incorporated into extracellular matrix structures isolated from rat liver (Hixson *et al.*, 1984), raise the possibility that DPP IV may play an important role in matrix assembly.

Other studies have reported that various tripeptide substrates of DPP IV slow the rate of hepatocyte and fibroblast spreading on matrices of fibronectin and/or collagen (Hanski *et al.*, 1985, 1988; Bauvois, 1988). Although these adhesion experiments were performed under different conditions than we used to measure binding (i.e. temperature), the results may be related to the finding that DPP IV inhibitors stimulate fibronectin binding to hepatocytes in suspension, particularly be-

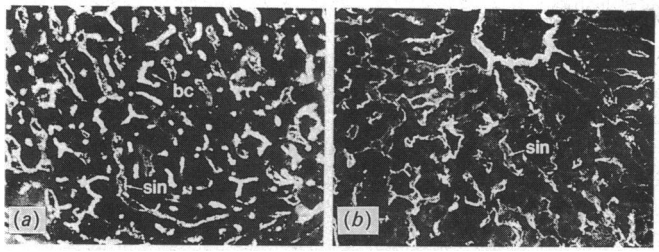

Fig. 5. Immunofluorescence localization of DPP IV (*a*) and fibronectin (*b*) in rat liver sections

Arrows show sinusoidal (sin) and bile canalicular (bc) hepatocyte membrane domains. Magnification × 93.

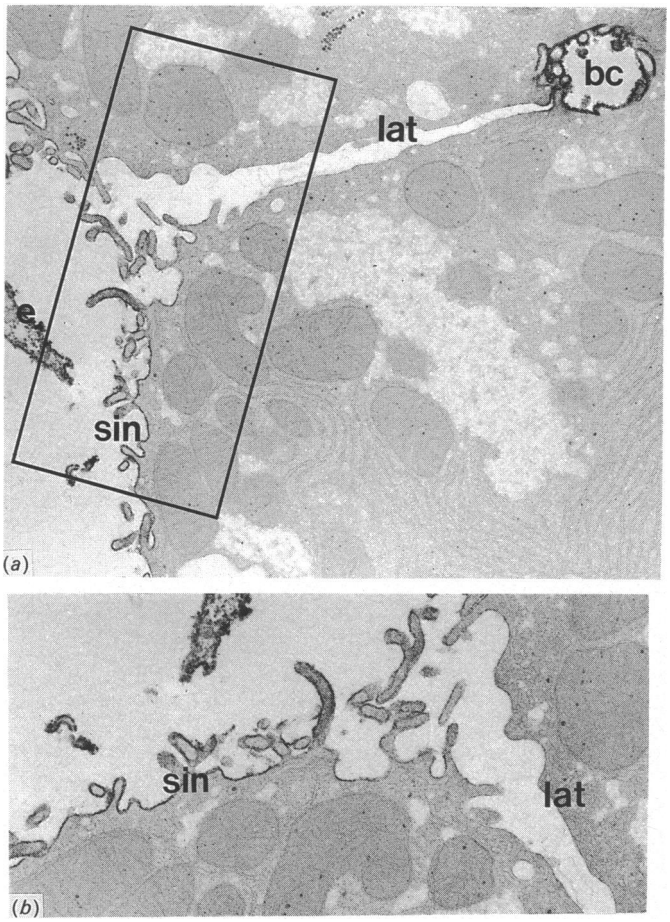

Fig. 6. Immunoperoxidase electron microscopy localization of DPP IV in mechanically-disrupted rat hepatocytes

Sinusoidal (sin), bile canalicular (bc) and lateral (lat) hepatocyte membrane domains are shown, as are endothelial cells (e). Panel (*b*) shows a higher magnification view of the area enclosed by the rectangle in panel (*a*). (*a*) × 11035, (*b*) × 16550.

cause in both experiments the effect of the inhibitors was most apparent during interactions of fibronectin with the hepatocyte surface. If this were the case, these observations together suggest the possibility that DPP IV binding to fibronectin results in a loss or alteration of the adhesive properties of fibronectin. We have observed that preparations of purified DPP IV (which could not cleave Z-Gly-Pro-MNA) induced a limited endoproteolytic cleavage of fibronectin (Piazza *et al.*, 1988). Further studies are, however, necessary to determine if this activity is related to its fibronectin-binding properties that we describe here.

Immunolocalization of DPP IV and fibronectin in rat liver

Localization studies of DPP IV and fibronectin in normal rat liver sections were performed to further investigate the possibility that DPP IV plays a role in fibronectin-mediated interactions of hepatocytes with extracellular matrix. As shown in Fig. 5(*a*), DPP IV was localized predominantly in bile canalicular membrane domains, but was also present in significant amounts in sinusoidal membranes. No staining was evident in lateral membranes. To clearly demonstrate that the sinusoidal localization of DPP IV was associated with hepatocytes, perfused–fixed, mechanically disrupted hepatocytes were labelled with MAb 236.3 by indirect immunoperoxidase techniques and examined by electron microscopy. As shown in Fig. 6, a significant portion of the sinusoidal reactivity was derived from DPP IV present on the sinusoidal membrane of hepatocytes. In addition, the entire periphery of endothelial cell membranes was strongly labelled with MAb 236.3. The presence of DPP IV on the sinusoidal membrane of hepatocytes and on endothelial cells is thus consistent with a role for this molecule in formation of the sinusoidal matrix.

Fibronectin antibodies displayed strong reactivity in the sinusoidal membranes with no labelling in either the lateral or bile canalicular membrane domains as shown in Fig. 5(*b*). This observation is consistent with previous reports describing the sinusoidal localization of fibronectin in rat liver (Martinez-Hernandez, 1984). The presence of both fibronectin and DPP IV along the sinusoidal membrane domain of hepatocytes lends further support to an involvement of DPP IV during interactions of hepatocytes with extracellular matrix *in vivo*.

Summary

In summary, we have shown by studies *in vitro* using purified DPP IV a direct binding of hepatocyte DPP IV to fibronectin. The fibronectin-binding domain on DPP IV appears to be distinct from its exoprotease catalytic site, since specific inhibitors of DPP IV noticably enhanced this interaction, possibly as a result of an altered conformation of DPP IV. Presumably by the same mechanism, DPP IV inhibitors also enhanced high-affinity binding of fibronectin to isolated hepatocytes. Localization studies of DPP IV and fibronectin in rat liver sections revealed that both proteins were present on the sinusoidal membrane of hepatocytes. Based on these results, we proposed a role of DPP IV in fibronectin-mediated interactions of hepatocytes with extracellular matrix and possibly matrix assembly. Putative exoproteolytic substrates and/or inhibitors of DPP IV, which have yet to be identified in the liver, may play a modulatory role in these processes.

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