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Extraenzymatic functions of the dipeptidyl peptidase IV-related proteins DP8 and DP9 in cell adhesion, migration and apoptosis

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Database
Dipeptidyl peptidase 8 (AF221634; Swiss-Prot Q9HBM5); dipeptidyl peptidase 9 (AY374518; Swiss-Prot Q6UAL0); dipeptidyl peptidase IV GenBank P27487; fibroblast activation protein GenBank U09278.

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The dipeptidyl peptidase IV gene family contains the four peptidases dipeptidyl peptidase IV, fibroblast activation protein, dipeptidyl peptidase 8 and dipeptidyl peptidase 9. Dipeptidyl peptidase IV and fibroblast activation protein are involved in cell–extracellular matrix interactions and tissue remodeling. Fibroblast activation protein is upregulated and dipeptidyl peptidase IV is dysregulated in chronic liver disease. The effects of dipeptidyl peptidase 8 and dipeptidyl peptidase 9 on cell adhesion, cell migration, wound healing and apoptosis were measured by using green fluorescent protein fusion proteins to identify transfected cells. Dipeptidyl peptidase 9-overexpressing cells exhibited impaired cell adhesion, migration in transwells and monolayer wound healing on collagen I, fibronectin and Matrigel. Dipeptidyl peptidase 8-overexpressing cells exhibited impaired cell migration on collagen I and impaired wound healing on collagen I and fibronectin in comparison to the green fluorescent protein-transfected controls. Dipeptidyl peptidase 8 and dipeptidyl peptidase 9 enhanced induced apoptosis, and dipeptidyl peptidase 9 overexpression increased spontaneous apoptosis. Mechanistic investigations showed that neither the catalytic serine of dipeptidyl peptidase 8 or dipeptidyl peptidase 9 nor the Arg-Gly-Asp integrin-binding motif in dipeptidyl peptidase 9 were required for the impairment of cell survival, cell adhesion or wound healing. We have previously shown that the *in vitro* roles of dipeptidyl peptidase IV and fibroblast activation protein in cell–extracellular matrix interactions and apoptosis are similarly independent of catalytic activity. Dipeptidyl peptidase 9 overexpression reduced β-catenin, tissue inhibitor of matrix metalloproteinases 2 and discoidin domain receptor 1 expression. This is the first demonstration that dipeptidyl peptidase 8 and dipeptidyl peptidase 9 influence cell–extracellular matrix interactions, and thus may regulate tissue remodeling.

Cell adhesion and migration, proliferation and apoptosis are central to many pathological processes involving tissue remodeling, including liver fibrosis, inflammation, angiogenesis, cancer growth and metastasis. The multifunctional glycoprotein dipeptidyl peptidase IV (EC 3.4.14.5) (DPIV) interacts with the extracellular matrix (ECM). DPIV is a ubiquitous aminopeptidase that has a variety of roles in the fields of metabolism, immunology, endocrinology and cancer biology [1–3]. We have shown that DPIV and its closest relative,

Abbreviations
CFP, cyan fluorescent protein; DP, dipeptidyl peptidase; DDR, discoidin domain receptor; DMEM, Dulbecco's modified Eagles's medium; ECM, extracellular matrix; FAP, fibroblast activation protein; GFP, green fluorescent protein; PI, propidium iodide; RAE, arginine-alanine-glutamine; RGD, arginine-glycine-asparagine; STS, staurosporine streptomyces; TIMP, tissue inhibitor of matrix metalloproteinase; YFP, yellow fluorescent protein.

fibroblast activation protein (FAP), exhibit altered expression in chronic liver injury [4,5] and that FAP expression correlates with human liver fibrosis severity [6]. Dipeptidyl peptidase 8 (DP8) and dipeptidyl peptidase 9 (DP9) are recently cloned proteinases of the DPIV gene family. DP8 and DP9 are closely related peptidases of 61% amino acid identity, and are ubiquitously expressed cytoplasmic molecules [7–9].

The functions of DP8 and DP9 are unknown. The known characteristics of DPIV and FAP may provide hypotheses concerning DP8 and DP9 function. DPIV is predominantly expressed on epithelial cells. DPIV binds fibronectin [10], and this interaction is independent of its enzymatic ability [11,12]. We recently showed that DPIV overexpression in HEK293T cells reduces cell migration and enhances induced apoptosis [12]. These DPIV–ECM interactions probably underlie some DPIV actions. DPIV expression is progressively downregulated as endometrial adenocarcinoma and ovarian carcinoma develop [13,14]. DPIV overexpression in melanoma and non-small cell lung carcinoma cell lines inhibits the processes of tumor progression, including anchorage-independent growth, cell migration and tumorigenicity [15,16]. Thus, the observed variability of DPIV expression levels in human tumors seems to relate to tumor invasiveness, proliferation and/or apoptosis.

FAP is a peptidase and gelatinase [4,17] expressed by mesenchymal cells. FAP associates with $\alpha_3\beta_1$ integrin on activated cells [18]. We recently showed that FAP overexpression in the LX-2 stellate cell line increases cell adhesion and migration and enhances induced apoptosis [12].

DP9 contains the Arg-Gly-Asp (RGD) cell attachment sequence [8], which is the best characterized integrin-binding motif, but it is difficult to envisage a role for this motif on a cytoplasmic protein. In this first investigation of DP8 and DP9 nonenzymatic functions, the hypothesis that DP8 and DP9 influence cell–ECM interactions was examined. In order to seek correlations between cell behaviors and peptidase expression levels, DP8 and DP9 overexpression in transfected cells was quantified by the expression of green fluorescent protein (GFP) fusion proteins. This approach minimizes the behavioral prejudices that are exhibited by stably transfected clones because they are selected for adherence, survival and proliferation. We found that, like cells that overexpress DPIV and FAP, cells overexpressing DP8 and DP9 exhibit behavioral changes in the presence of ECM components. We have demonstrated that these effects are independent of enzyme activity and of the RGD motif in DP9.

Results

Specific recombinant expression of DP8 and DP9

AD293 or 293T cells transfected with DP8 and DP9 showed consistent high-level transfection (Fig. 1A,B; supplementary Fig. 1) and significant specific DP activity, shown by fourfold to sixfold greater D_{450} than untransfected cells (Table 1). Mutation of the catalytic serine ablated activity; DP9 data are given in Table 1, and DP8 was assessed by cell stain (not shown). DP8 and DP9 have been localized to Golgi and endoplasmic reticulum [7,8]. Concordantly, in the 293T cells transfected with DP8–GFP and DP9–GFP, the fluorescence was localized to the cytoplasm (supplementary Fig. 1). The 293T cell line lacks FAP and expresses DPIV only intracellularly and at low levels [12]. Neither DP8 or DP9 transfection altered FAP or DPIV expression in comparison to untransfected 293T cells (Fig. 1C–F).

DP9 overexpression impaired *in vitro* cell adhesion

Cells expressing DP9–GFP but not those expressing DP8–GFP exhibited about 20% less cell adhesion on plastic coated with collagen I, fibronectin or Matrigel than cells expressing GFP alone ($P < 0.05$) (Fig. 2A). Flow cytometry showed that markedly more DP9–GFP-high-expressing and GFP-high-expressing cells were present among the nonadherent than the adherent cell population (Fig. 2B–E).

DP8 and DP9 reduced migration into monolayer wounds

In vitro wound healing assays indicate whether cells overexpressing a protein differ in their ability to repopulate a small area of coated plastic surface from which the cell monolayer has been scraped off. This is an assay of cell migration rather than proliferation [19]. Cells transfected with DP8–GFP and those transfected with DP9–GFP exhibited reduced migration into wounds on collagen-coated and fibronectin-coated surfaces (Fig. 3A), indicating an ability of DP8 and DP9 overexpression to impair monolayer wound healing on ECM.

DP8 and DP9 impaired cell migration

Cell migration was also assessed in transwells. *In vitro* cell migration assays showed that cells expressing DP8–GFP exhibited reduced migration towards colla-

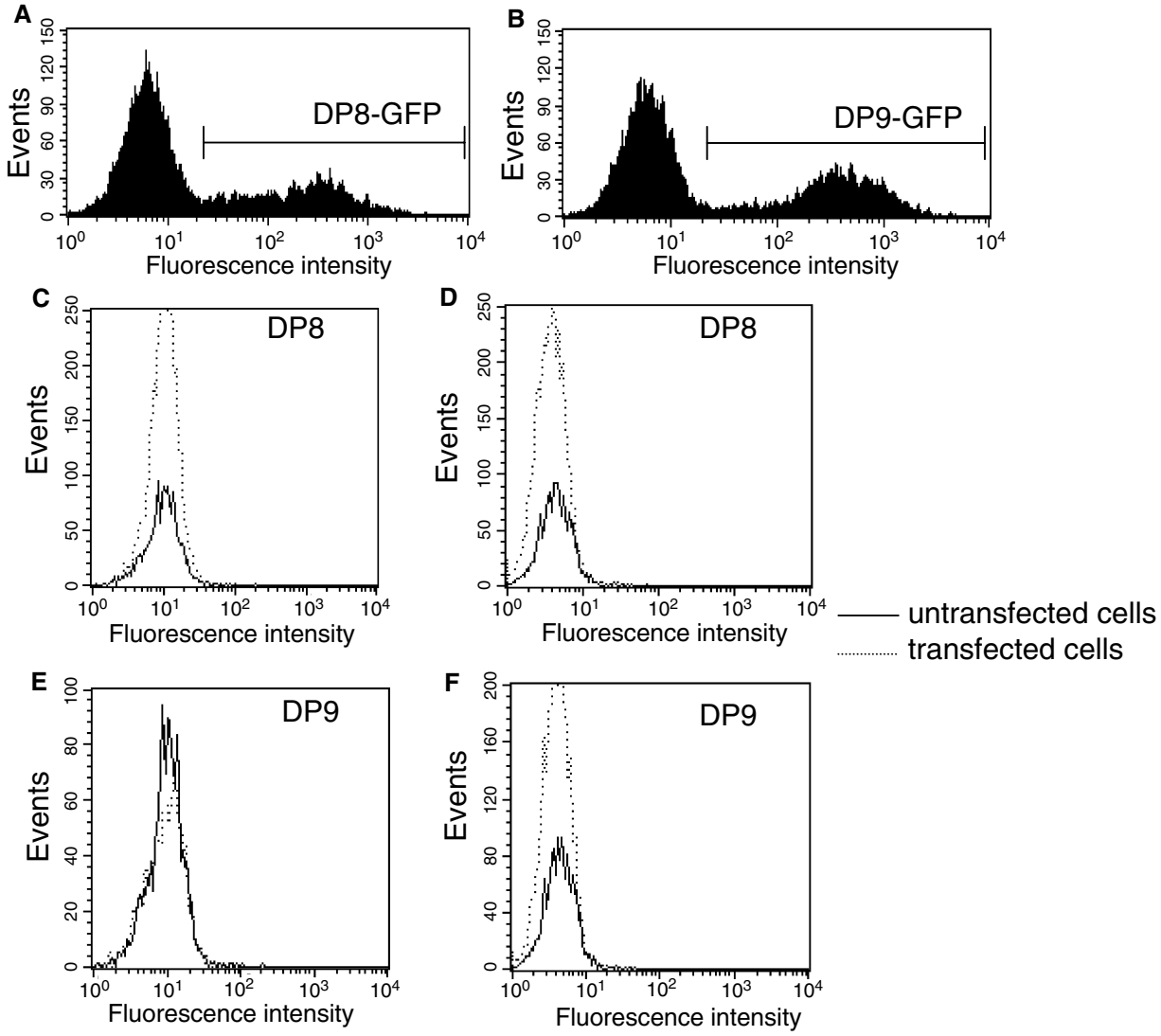

Fig. 1. Specific recombinant expression of dipeptidyl peptidase 8 (DP8) and dipeptidyl peptidase 9 (DP9). Flow cytometry showed expression of DP8–green fluorescent protein (GFP) (A) and DP9–GFP (B) by transfected AD293 cells. Potent antibodies to dipeptidyl peptidase IV (DPIV) (C, E) and fibroblast activation protein (FAP) (D, F) were used to show that DPIV and FAP levels were not altered in DP8–GFP-transfected (C, D) and DP9–GFP-transfected (E, F) cells compared to untransfected control 293T cells. These analyses show data from all live cells. To demonstrate that the method could detect DPIV and FAP, DPIV-transfected and FAP-transfected cells were shown to be intensely immunopositive when stained with their homologous antibodies (not shown).

gen I across the transwell membrane in comparison to the GFP-expressing controls (Fig. 4). DP9–GFP-expressing cells exhibited less migration towards collagen I, fibronectin or Matrigel.

Peptidase activity and the RGD motif were not required for DP9-dependent impairment of cell adhesion

To investigate the mechanism of DP9-dependent impairment of cell adhesion, an enzyme-negative mutant of DP9–GFP, in which the catalytic serine was replaced with alanine, was evaluated. In addition, the RGD motif of DP9 was replaced with Arg-Ala-Glu (RAE) to investigate whether this integrin-binding motif played a role. The RGD integrin-binding motif was first identified in fibronectin and is not known to have a cytoplasmic role. As DP9 is cytoplasmic, the DP9 RGD was expected not to influence cell–ECM interactions. The RAE mutant retained peptidase activity, whereas the Ser → Ala mutant had very low activity (Table 1). Neither the DP9 enzyme-negative

Table 1. Peptidase assays of transfected cells using the chromogenic substrate H-Ala-Pro-pNA (A) or the fluorogenic substrate H-Ala-Pro-AFC (B). DP8, dipeptidyl peptidase 8; DP9, dipeptidyl peptidase 9; RAE, Arg-Ala-Glu.

(A)

Transfected gene	$\Delta D_{405\ nm}\cdot min^{-1}$
DP8	0.462 ± 0.007
DP9	0.327 ± 0.001
DP9 RAE	0.241 ± 0.005
DP9 E–	0.152 ± 0.004
Untransfected cells	0.078 ± 0.005

(B)

Transfected gene	Δ fluorescence$\cdot min^{-1}$
DP8–V5–His	76.5 ± 2.7
DP9–V5–His	101.6 ± 1.02
Untransfected cells	17.4 ± 1.07
V5–His control	22.3 ± 1.93

mutant nor the RGD → RAE mutant differed from wild-type DP9 in impairing cell adhesion (Fig. 5A).

Peptidase activity and the DP9 RGD motif were not required for DP8-dependent or DP9-dependent impairment of wound healing

The effects of DP8–GFP and DP9–GFP enzyme-inactive mutants and the DP9–GFP RGD → RAE mutant on wound healing were investigated (Fig. 5B,C). We found that in the conditions tested, i.e. on a collagen I-coated or fibronectin-coated surface, the mutants behaved similarly to wild-type controls. These data indicated that the effects on wound healing were independent of enzyme activity and the DP9 RGD motif.

DP8 and DP9 overexpression increased stuarosporine streptomyces (STS)-induced apoptosis

We investigated whether some of the effects seen on wound healing, cell migration and cell adhesion might be in part related to apoptotic or proliferative effects. In particular, loss of adhesion can promote apoptosis [20]. In time-course experiments, both DP8–CFP-transfected and DP9–CFP-transfected cells exhibited increased STS-induced apoptosis in comparison to cells transfected with cyan fluorescent protein (CFP) alone (Fig. 6). Furthermore, the same effect was seen with use of the enzyme-negative mutants DP8–GFP Ser739 → Ala or DP9–GFP Ser729 → Ala, or the DP9 RGD → RAE mutant, indicating that this effect was independent of enzyme activity or the RGD motif.

Interestingly, even without STS treatment there were increases of about 20–25% in the percentages of apoptotic cells in the cell subpopulations that were overexpressing any of the three DP9 constructs. The extent of increased apoptosis among DP9-expressing cells was similar to the extent of the adhesion deficit. This concordance of apoptosis and adhesion suggests that one may cause the other.

In the proliferation studies we used cells transfected with V5–His fusion constructs and compared them with vector-transfected cells, as well as using the GFP constructs. Transfection with DP8–GFP or DP9–GFP produced proliferation rates greater than those obtained with GFP transfection (Table 2). However, cells transfected with DP8–V5–His or DP9–V5–His showed no significant differences from those transfected with vector only. Transfection efficiencies of V5–His constructs were about 35%, comparable to those of GFP constructs. In this assay, GFP expression was associated with decreased proliferation [12]. The DP8–GFP and DP9–GFP fusion proteins had smaller effects on proliferation but this may not be biologically significant.

Apoptotic DP9-positive cells in the wound-healing assay

The increased apoptosis of DP9-expressing cells may contribute to their reduced migration into monolayer wounds. In wounded monolayers, greater numbers of DP9-positive cells were propidium iodide (PI) positive in wound than in nonwound regions (Fig. 7). Fewer PI-positive cells were seen in GFP-transfected monolayers. Thus, apoptosis possibly contributed to the reduced numbers of DP9-positive cells in monolayer wounds.

The actin cytoskeleton was unaffected by DP8 or DP9 overexpression

We investigated whether DP8 or DP9 overexpression was associated with changes in the actin cytoskeleton as a mechanism for altering cell adhesion and migration. High-magnification, high-resolution confocal microscopy showed that DP8 was visible throughout the cytoplasm (Fig. 8A), whereas DP9 was more localized (Fig. 8B). There was little or no colocalization of DP8 or DP9 with phalloidin-labeled actin cytoskeleton in AD293 cells plated on slides coated with collagen I, fibronectin or Matrigel. These data suggested no association between DP8 or DP9 and the actin cytoskeleton.

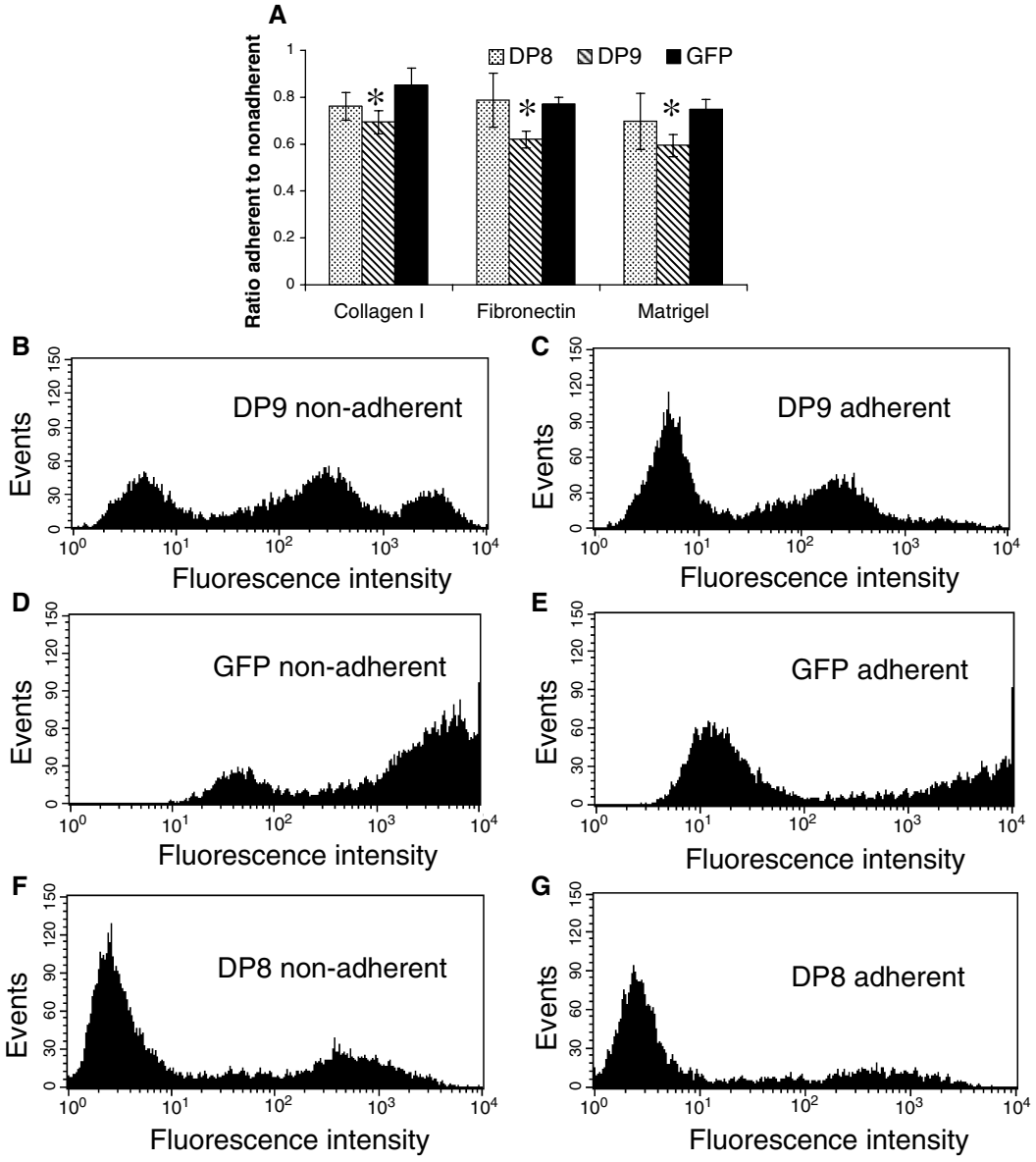

Fig. 2. Dipeptidyl peptidase 9 (DP9)–green fluorescent protein (GFP) overexpression decreased cell adhesion. *In vitro* cell adhesion of cells transfected with dipeptidyl peptidase 8 (DP8)–GFP, DP9–GFP and GFP control is expressed as a ratio of the percentage of fluorescent cells in the adherent population to the percentage of fluorescent nonadherent cells (A). Flow cytometry profiles of the nonadherent (B, D, F) and adherent (C, E, G) DP9–GFP+ (B, C), GFP+ (D, E) and DP8–GFP+ (F, G) live cell populations show that the nonadherent populations contained more high-expressing cells, but this was less marked in the DP8–GFP profile.

Molecular phenotyping of 293T cells overexpressing DP8 and DP9

We investigated whether cells overexpressing DP8 and DP9 demonstrated changes in expression levels of an extensive panel of proteins associated with cell adhesion. Discoidin domain receptor 1 (DDR1) is a non-integrin collagen receptor that stimulates adhesion and migration [21]. The antibody to DDR1 is specific for an epitope in its cytoplasmic domain. Increased expression of E-cadherin and tissue inhibitor of matrix metalloproteinase 2 (TIMP2) by DPIV-transfected cells has been reported [22]. β-Catenin associates with E-cadherin and influences cell adhesion [23]. Cytoplasmic levels of DDR1, E-cadherin and TIMP2 were reduced in DP9–CFP-overexpressing cells compared to CFP-overexpressing or DP8–CFP-overexpressing cells (Table 3, Fig. 9A). Both DP8-overexpressing and

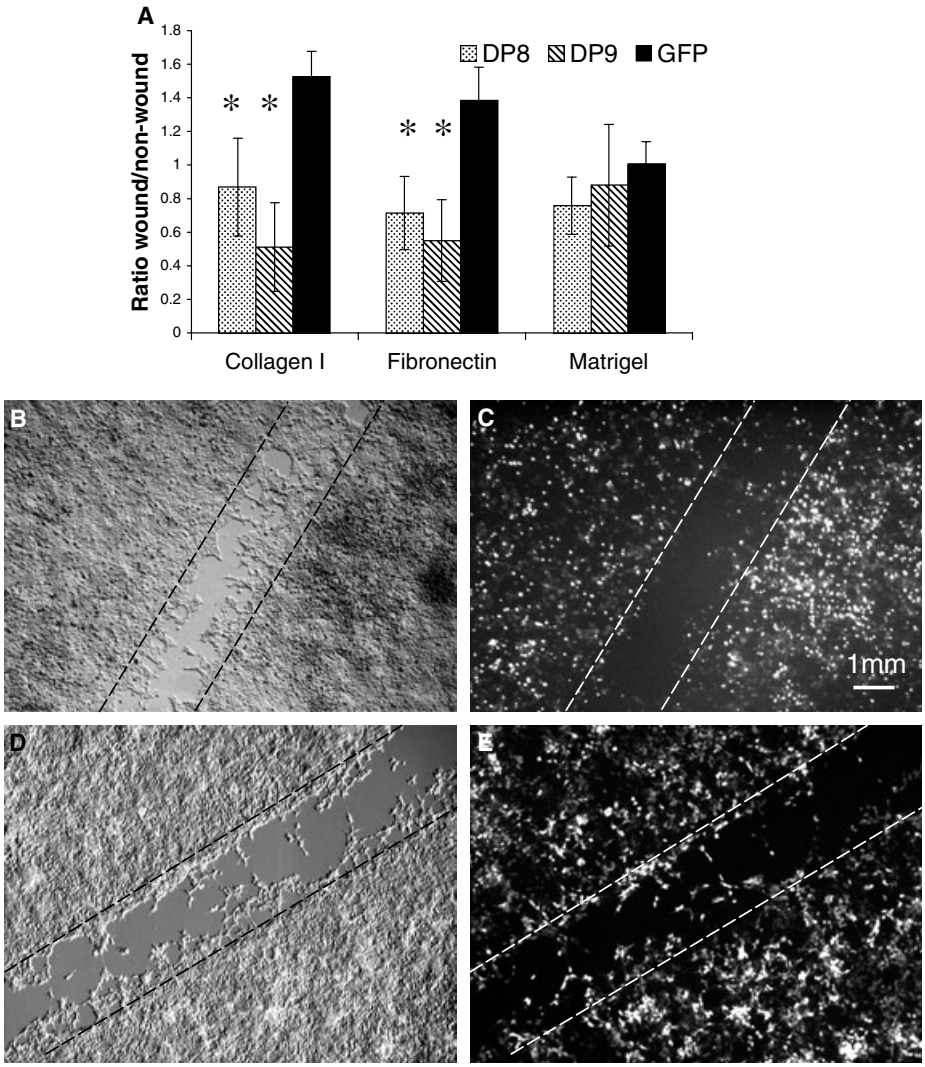

Fig. 3. Dipeptidyl peptidase 8 (DP8)–green fluorescent protein (GFP) and dipeptidyl peptidase 9 (DP9)–GFP reduced *in vitro* wound healing. Ratios of the percentage of fluorescent cells in the wound area to the percentage of fluorescent cells in nonwound regions of the monolayer on the same extracellular matrix (ECM) substrate (A) (mean ± SD). Bright field image of DP9–GFP-transfected cells in a wounded monolayer, representing the location of all cells (B). Identical field, GFP fluorescence image, revealing that fewer fluorescent cells reside in the wound area (C). Similarly, GFP-transfected cells in one field of a wounded monolayer are shown in bright field (D) and in a fluorescence image (E). Dashed lines border the wound area.

DP9-overexpressing cells contained less β-catenin (Table 3, Fig. 9B).

Discussion

This is the first report on the biological significance of DP8 and DP9. A portfolio of cell–ECM interaction assays indicated roles for DP9 in cell adhesion, *in vitro* wound healing, cell migration and apoptosis, and for DP8 in wound healing, cell migration and apoptosis enhancement (Table 4). DP9 overexpression impaired cell behavior with regard to a wider range of ECM components than did DP8 overexpression, in that no effects were seen for DP8 on Matrigel. Despite their close sequence relatedness, DP8 and DP9 exert these differences in their cellular effects. Therefore, these two proteins are likely to have different functions and ligands.

These data indicate that DP8 and DP9 have some overlapping properties with DPIV as well as FAP, a DPIV family member that is expressed only in diseased and damaged tissue and in tissue remodeling [12].

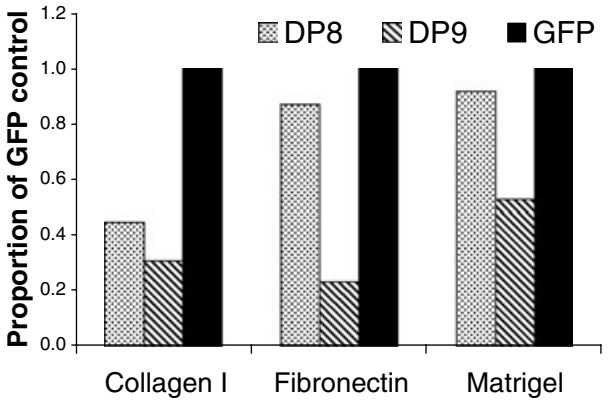

Fig. 4. Cell migration is reduced by overexpression of dipeptidyl peptidase 9 (DP9) or dipeptidyl peptidase 8 (DP8). *In vitro* migration of 293T cells transfected with DP8–green fluorescent protein (GFP), DP9–GFP and GFP control across transwells towards extracellular matrix (ECM) components. Each ratio of GFP-derived fluorescence-positive (GFP+) cells in the upper chamber to GFP+ cells in the lower chamber was normalized to the ratio obtained from GFP control-transfected cells.

DPIV-transfected LOX melanoma cells in the presence of Matrigel have reduced invasiveness compared to controls [24]. DPIV-transfected non-small cell lung carcinoma cells have shown inhibition of cell migration, increased apoptosis, inhibition of anchorage-independent growth and suppression of tumor growth in nude mice [16]. Our own studies on DPIV and FAP in HEK 293T and LX-2 cells have further established these roles in cell–ECM interactions [12].

Cell adhesion is crucial in monolayer wound healing and cell migration. Therefore, the adhesion defect of cells overexpressing DP8 or DP9 may contribute to the observed defects in wound healing and cell migration. Moreover, loss of adhesion can promote apoptosis [20]. Therefore, the reduced adhesion of cells overexpressing DP9 may contribute to their increased apoptosis. Conversely, apoptotic cells possess reduced adhesive capacity. Our data also indicate that the increased spontaneous apoptosis of DP9-overexpressing cells probably contributes to their reduced cell migration. Determining the relative roles of adhesion and apoptosis is difficult. DP9 overexpression did not compromise cellular protein synthesis, as there was not

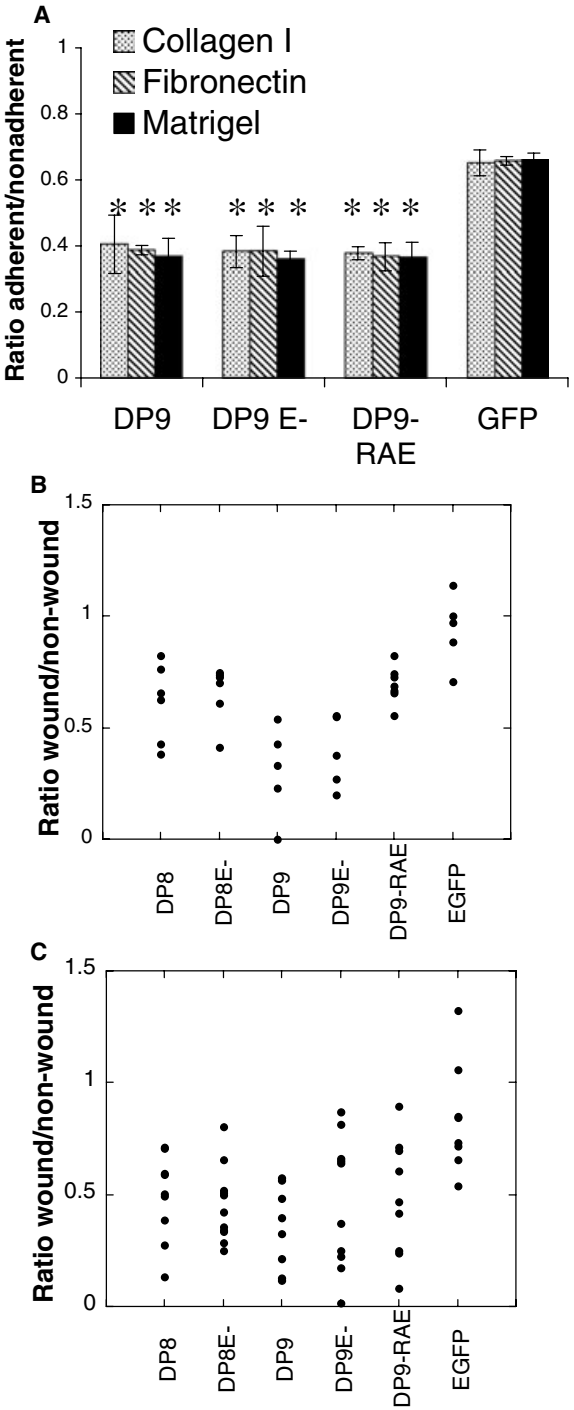

Fig. 5. The dipeptidyl peptidase 8 (DP8)-dependent and dipeptidyl peptidase 9 (DP9)-dependent impairment of adhesion and wound healing was independent of enzyme activity and the Arg-Gly-Asp (RGD) motif. The RGD integrin-binding motif was mutated out of DP9 to produce Arg-Gly-Asp28 → Arg-Ala-Glu–green fluorescent protein (GFP) (DP9 RGD → RAE). Enzyme-negative mutants of DP8 (DP8 E–) and DP9 (DP9 E–) were produced by replacement of the catalytic serine with alanine. (A) Cell adhesion was calculated as a ratio of the percentages of cells exhibiting GFP-derived fluorescence in the adherent and nonadherent cell populations (mean ± SD of triplicates). Wound healing of transfected 293T monolayers on (B) collagen I and (C) fibronectin indicated no significant difference between DP9 mutants and wild type.

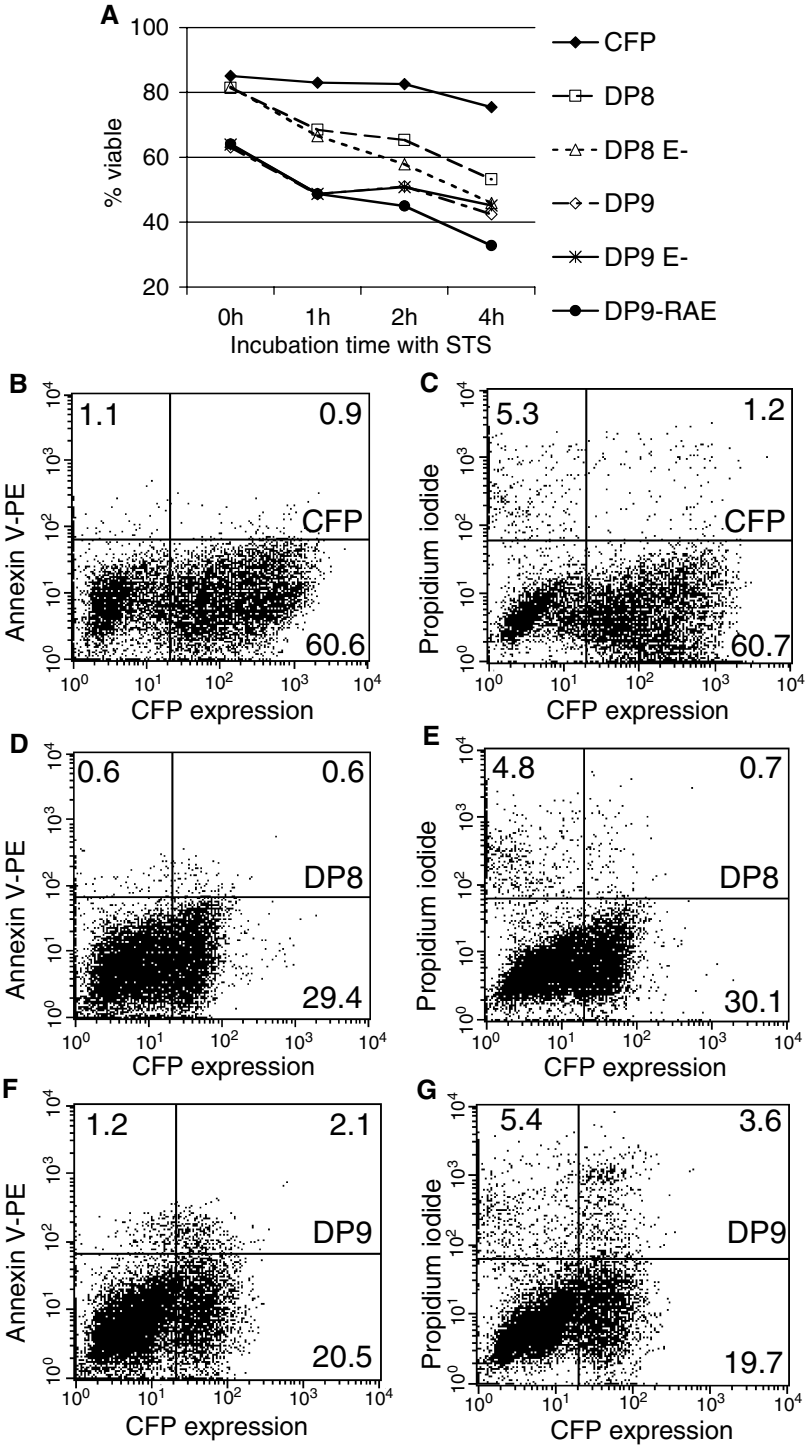

Fig. 6. Dipeptidyl peptidase 8 (DP8) and dipeptidyl peptidase 9 (DP9) enhanced staurosporine streptomyces (STS)-induced apoptosis independently of enzyme activity and the Arg-Gly-Asp (RGD) motif. (A) Cells transfected with wild-type and mutated DP8–cyan fluorescent protein (CFP) or DP9–CFP or CFP were exposed to STS at time zero, and the nonapoptotic cells were enumerated by flow cytometry. Percentage viable is the percentage of cells that are CFP-derived fluorescence positive, annexin V negative and propidium iodide negative. Annexin V (B, D, F) and propidium iodide (C, E, G) flow cytometry scattergrams of CFP (B, C), DP8–CFP (D, E) and DP9–CFP (F, G). The percentage of positive cells is shown in each quadrant.

a universal decrease in protein expression by DP9-positive cells (Table 3).

We showed that the enzymatic activities of DP8 and DP9 are not required for their effects on adhesion, wound healing and apoptosis. Similarly, the enzyme activities of DPIV and FAP are not required for their cell–ECM interaction roles [12,15,16,24]. Thus, the mechanisms of action probably involve protein–protein interactions, which most likely occur on the β-propeller domains of these proteins [25]. No ligand of DP8 or DP9 has been reported. The multifunctional aspect of these molecules both as enzymes and as interacting

Table 2. Cell proliferation. A standard thymidine uptake assay was used. Results are expressed as a proliferation quotient, which is the ratio of counts·min^{-1} of transfected and untransfected cell populations from up to five transfection experiments. Statistical analyses compared each dipeptidyl peptidase 8 (DP8) and dipeptidyl peptidase 9 (DP9) fusion protein with the corresponding empty vector control. GFP, green fluorescent protein.

Transfected cDNA	Proliferation quotient (mean ± SD)	*P*-value (Mann–Whitney *U*-test)
DP8–GFP	0.67 ± 0.07	< 0.0001
DP9–GFP	0.54 ± 0.08	0.0016
GFP control	0.46 ± 0.09	
DP8–V5–His	0.90 ± 0.03	0.294
DP9–V5–His	0.96 ± 0.06	0.294
V5–His control	0.92 ± 0.06	

proteins highlights the need to understand their structure [1,2]. It also suggests that specific enzyme inhibitors of the DPIV family might not influence cell–ECM interactions. However, there are no known inhibitors specific for DP8 or DP9 that could be used to test this proposition.

Many cytoplasmic events are involved in cell–ECM interactions that lead to changes to cell behavior, so it is possible that cytoplasmic DP8 and DP9 influence such events. For example, integrin activation can be controlled by signaling pathways that involve protein–protein interactions [26]. Nischarin is cytoplasmic and interacts with the cytoplasmic tail of integrins, and thus influences cell migration [27]. Cytoskeletal changes were not observed in cells overexpressing DP8 or DP9, so these proteins probably do not directly bind to the actin cytoskeleton. However, the observed decreases in DP9-overexpressing cells of the ECM-interacting molecules DDR1, a kinase activated by collagen binding, and TIMP2, a matrix metalloproteinase inhibitor, suggest possible DP9 target pathways. TIMP2 and β-catenin can influence cell adhesion and apoptosis [23,28]. DDR1 is an integrin-independent cell adhesion molecule. DPIV reduces cell adhesion by dephosphorylating p38 MAP kinase and β_1-integrin [29], so the effects of DP8 and DP9 on p38, β_1-integrin and DDR1 phosphorylation require examination. Changes in TIMP2 and β-catenin expression may be secondary to effects on integrins and/or DDR1.

DPIV and FAP, although cell-surface molecules, are also cytoplasmically expressed and so may have similar cytoplasmic actions to DP8 and DP9. The recent discovery that cytoplasmic DPIV can be phosphorylated [30] supports this contention. Many potential phosphorylation sites in DP8 and DP9 can be identified using the NetPhos server [31] (data not shown). The

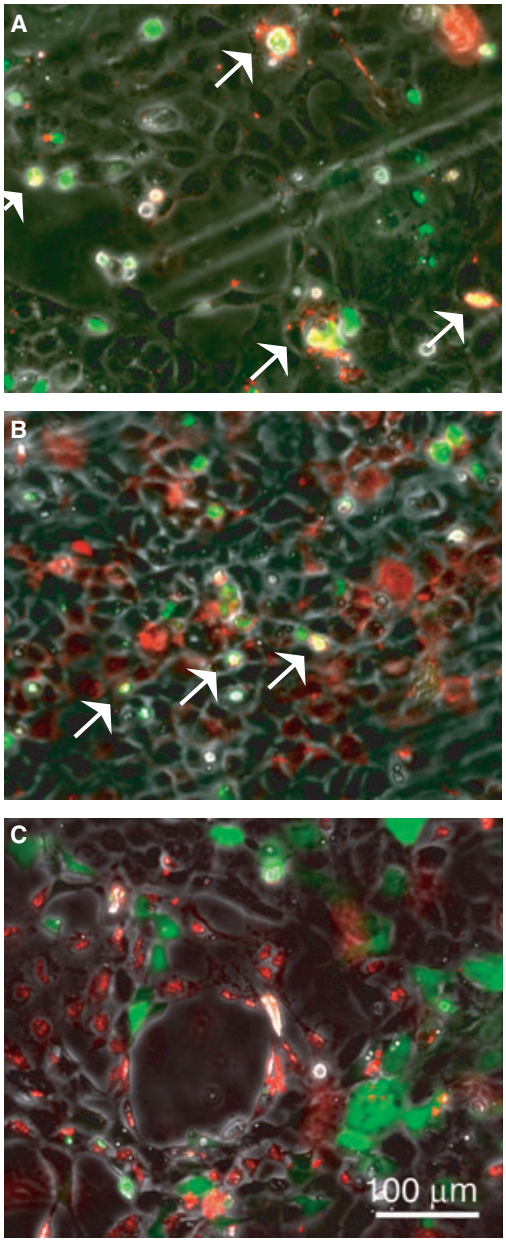

Fig. 7. Apoptotic dipeptidyl peptidase 9 (DP9)-expressing cells in wounded monolayers. Wounded monolayers had more apoptotic DP9-expressing cells than green fluorescent protein (GFP) control-expressing cells, and more apoptotic DP9-expressing cells in wound (A) than in nonwound (B) regions. A DP9–GFP-transfected (green) (A, B) and a wound of a GFP-transfected (green) (C) AD293 monolayer on collagen I. Propidium iodide-stained (red) dead/apoptotic cells.

cell-surface expression of DPIV and FAP probably has additional effects on cell behavior via fibronectin and integrin binding [10,18,29].

The increased STS-induced apoptotic effect of DP8 and DP9 may indicate that under certain biological

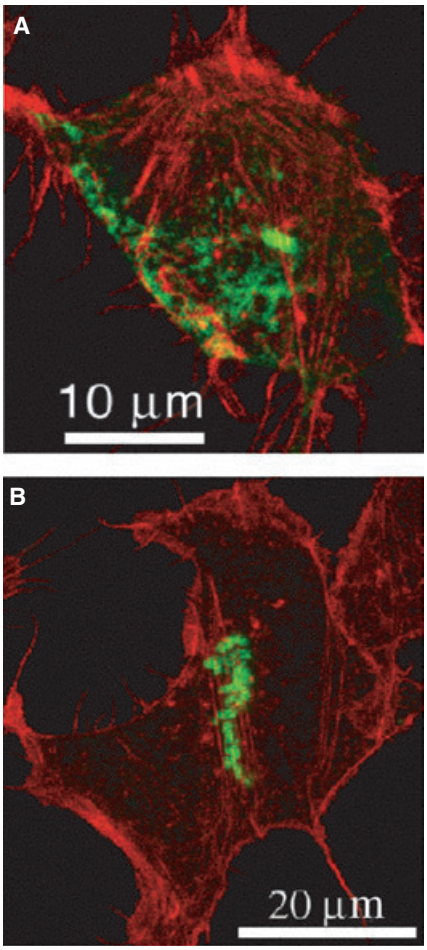

Fig. 8. Dipeptidyl peptidase 8 (DP8), dipeptidyl peptidase 9 (DP9) and the actin cytoskeleton. Phalloidin staining (red). (A) DP8–green fluorescent protein (GFP). (B) DP9–GFP-transfected AD293 cells with confocal imaging.

circumstances DP8 might enhance apoptotic effects. DPIV and FAP, like DP9, increase apoptosis [12,16, 32–34]. Apoptosis is an important process in tissue remodeling, including recovery from liver injury [35].

DP9 mRNA is ubiquitous and highly expressed in tumors [8]. The reduced migration by DP9-overexpressing cells towards collagen I and fibronectin in transwells suggests that DP9 might reduce cell migration in tumors and the injured liver. Thus, a function of increased DP9 expression may be to retain expressing cells in the tumor and in sites of expression in the injured liver. It would be interesting to localize the DP9-expressing cells in tumors and cirrhotic liver.

The biological significance of DP8 and DP9, as new DPIV family members, is largely unknown. This study is the first indication of some similarities as well as differences between DP8, DP9, DPIV and FAP in their cell biological roles [1,2]. All four proteins are involved in cell–ECM interactions and influence apoptosis, but DP8 did not influence adhesion and only DP9 acted as a primary trigger of apoptosis. DP8 and DP9 may also have *in vivo* roles as intracellular enzymes, with as yet unidentified natural substrates. It would be interesting to obtain direct evidence for DP8 and DP9 involvement in cancer, fibrosis and other tissue-remodeling processes.

Experimental procedures

Constructs and mutagenesis

The cDNAs of human DP8 and DP9 (GenBank accession numbers AF221634 and AY374518) were cloned in-frame upstream of C-terminal GFP, yellow fluorescent protein (YFP) and CFP in the vectors pEGFP-N1, pEYFP-N1 and pECFP-N1 (BD Biosciences Clontech, Palo Alto, CA). This was achieved by PCR of the insert with Platinum Pfx Taq (Invitrogen, Carlsbad, CA) and primers containing incorporated *Sal*I and *Kpn*I restriction sites and stop codon removal (Table 5).

Transformed, kanamycin-resistant plasmid DNA was purified from *Escherichia coli* DH5α cells (Invitrogen) and completely sequenced. Enzyme-negative mutants of DP8 and DP9 were generated using point mutation primers for

Table 3. The molecular phenotype of 293T cells overexpressing dipeptidyl peptidase 8 (DP8) and dipeptidyl peptidase 9 (DP9). Immunofluorescence flow cytometry. Median fluorescence intensities from transfected 293T cells, following subtraction of the median fluorescence intensity from each corresponding negative control. These results are from the live cyan fluorescent protein (CFP)-positive cells. MMP, matrix metalloproteinase; ND, not determined; DDR1, discoidin domain receptor 1; TIMP2, tissue inhibitor of matrix metalloproteinase 2.

Transfected cDNA	E-cadherin	β-catenin	MMP2	TIMP2	CD44	CD29	CXCR4	CXCL12	DDR1
Cell surface									
CFP	7.56	0.97	1.8	0.47	13.6	4.18	6.61	4.13	0.52
DP8–CFP	9.72	0.6	1.94	0.92	14	5.84	7.51	2.83	0.66
DP9–CFP	7.63	0.58	1.54	1.21	11.3	5.12	7.78	4.33	0.69
Permeabilized									
CFP	30.63	184	3.14	63.7	ND	ND	39.9	14.4	193
DP8–CFP	31	145	3.88	72.8	ND	ND	37	17.9	206
DP9–CFP	20.4	136	2	48.5	ND	ND	35	10	139

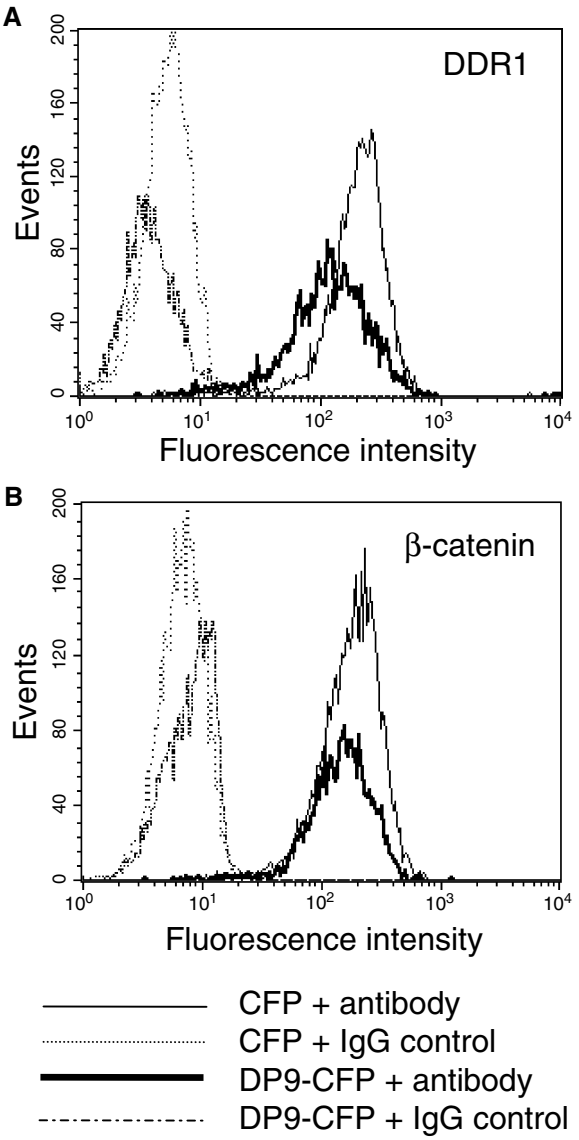

Fig. 9. Reduced discoidin domain receptor 1 (DDR1) and β-catenin levels in dipeptidyl peptidase 9 (DP9)-overexpressing cells. Flow cytometry of 293T cells permeabilized and then immunostained for DDR1 (A) or β-catenin (B) expression 40 h after transfection. Only cyan fluorescent protein (CFP) fluorescence-positive cells were analyzed. The reduced distances between antibody (solid lines) and control (broken lines) peaks from DP9–CFP-expressing cells indicate reduced expression levels of DDR1 and β-catenin.

alanine replacement of the catalytic serine residues of DP8 at position 739 and of DP9 at position 729 [36]. The RGD → RAE sequence substitution that ablates integrin binding [37] was engineered into DP9 using point mutation primers for alanine replacement of glycine at position 12 and glutamine replacement of asparagine at position 13.

All constructs were fully sequenced and tested for enzyme activity on 2×10^4 transfected whole cells permeabilized in 0.1% Tween-20/NaCl/P_i pH 7.4 using the chromogenic substrate H-Ala-Pro-*p*-nitroanilide (Bachem, Bubendorf, Switzerland), and measuring absorbance at 405 nm, or the fluorescent substrate H-Ala-Pro-AFC (Bachem) on a Victor2 plate reader (Wallac) (Table 1) or by staining cells using Ala-Pro-4-methoxy-β-naphthylamide-HCl (Sigma, St Louis, MO) [8]. Plasmid DNA extraction, site-directed mutagenesis, human embryonic kidney (HEK) 293T and AD293 cell lines (ATCC, CRL-11268), pcDNA3.1/V5/HisA vector constructs, transfection and enzyme activity assays have been described previously [8,36,38]. AD293 cells are a more adhesive variant of HEK293.

Cell adhesion assay

The cell adhesion assay was carried out as previously described [12]. That is, 40 h after transfection, 293T cells were plated on rat-tail collagen I (Sigma), human fibronectin (Sigma) or Matrigel (BD Biosciences Discovery Labware, Bedford, MA). Following incubation for 10 min at 37 °C, nonadherent cells were gently separated from adherent cells and individually analysed for percentages of GFP-expressing cells by use of flow cytometry [38].

In vitro wound-healing assay

The wound-healing assay was performed as described [12]. 293T cells were plated onto plastic coated with collagen I, fibronectin or Matrigel. Forty hours after plating, the monolayer was scraped with a fine pipette tip to produce wounds of about 8 mm × 1 mm, and then 1% fresh fetal bovine serum was added. Images were obtained after 24–48 h of further incubation. KS400 image analysis software version 3.0 (Zeiss, Heidelberg, Germany) with automatic threshold and lowpass filter was used to count migrated cells by measuring the total area covered by cells (bright field) and the area covered by fluorescence-positive cells in the wound and nonwound portions of each image.

Table 4. Data summary. N, no significant effect; ↑, increase; ↓, decrease.

Gene	Adhesion	Migration	Wound healing	Proliferation	Apoptosis	STS-induced apoptosis
DP8	N	↓Collagen	↓Collagen ↓Fibronectin	N	N	↑
DP9	↓Collagen ↓Fibronectin ↓Matrigel	↓Collagen ↓Fibronectin ↓Matrigel	↓Collagen ↓Fibronectin	N	↑	↑

Table 5. Primers used for generating green fluorescent protein (GFP) fusion constructs and for mutagenesis.

Primer	Nucleotide sequence	Size
GFP fusion		
DP8SalIfus.For	ATT TAT (GTC GAC) AAT GCA ACA TGG CAG CAG CAA TG	35 mer
DP8KpnIfus.Rev	CGA TCT (GGT ACC) CCT CTA GAT ATC ACT TTT AGA GC	35 mer
DP9EcoRI.For	ATA TAT GAA TTC AGG ATG GCC ACC ACC GGG	30 mer
DP9SalI.Rev	GGG CCC GTC GAC TGC AAG AGG TAT TCC TGT AG	32 mer
Mutagenesis		
DP8S739 AFor	CCA CGG CTG GGC CTA TGG AGG ATA C	25 mer
DPP8S739 ARev	GTA TCC TCC ATA GGC CCA GCC GTG G	25 mer
DP9RGDmut.For	GCC GAC CGA GCC GAA GCA GCC GCC	24 mer
DP9RGDmut.Rev	GGC GGC TGC TTC GGC TCG GTC GGC	24 mer
DP9S729 AFor	CCA TGG CTG GGC CTA CGG GGG CTT C	25 mer
DP9S729 ARev	GAA GCC CCC GTA GGC CCA GCC ATG G	25 mer

Cell migration assay

The cell migration assay was performed as described [12]. Transwell inserts (BD Biosciences Discovery Labware) were protein-coated on the lower side, and 40 h after the overnight transfection, serum-starved 293T cells were placed in the upper chamber. The lower chamber contained conditioned medium with an additional 1% fetal bovine serum. Medium (10% fetal bovine serum in Dulbecco's modified Eagle's medium (DMEM) was conditioned by overnight exposure to confluent 293T cell monolayers. After a 3-day incubation, cells were harvested and the percentages of fluorescent cells were determined by flow cytometry.

Apoptosis

As described previously [12], 293T cells were transfected with CFP fusion constructs, replated 30 h later, and on the next day treated with 4 μM STS (Sigma, St Louis, MO) for 2 h, 4 h or 6 h. STS is a chemotherapeutic agent that induces cellular apoptosis. Annexin V (1 : 50) and PI (100 ng·mL^{-1}) were used to enumerate the apoptotic CFP-positive cells by flow cytometry.

Proliferation assay

Proliferation was quantified by a standard thymidine uptake assay. 293T cells were transfected with DP8 or DP9 fusion constructs or empty vector. Cells were harvested 24 h after transfection, and 4–6 replicates of 3000 cells per well incubated for 5 h before addition of tritiated thymidine (Perkin-Elmer Life Sciences, Boston, MA) at 0.5 μCi per well. A further 19 h later, cells were harvested with a Skatron cell harvester onto a glass fibre filter (Wallac, Turku, Finland) and the incorporation of tritiated thymidine determined in a Wallac 1205 Betaplate^{TM} liquid scintillation counter. Confirmation of efficient transfection with pcDNA3.1/V5/HisA-derived constructs was ascertained by flow cytometry of transfected cells stained with anti-V5 monoclonal antibody (Invitrogen) and enzyme activity assays [38].

Molecular phenotyping of overexpressing cells

Immunofluorescence flow cytometry has been described previously [36]. Antibodies and phalloidin and their working dilutions are listed elsewhere [12]. For immunocytochemistry, transfected cells were incubated overnight on collagen-coated chamber slides (Nunc, Naperville, IL), formalin-fixed and permeabilized, and then imaged with a Radiance Plus Confocal Scanning System (Bio-Rad, Hercules, CA) and LASERSHARP 2000 software.

Statistics

Each experiment was repeated three to six times. Results are expressed as means ± standard deviation. Differences among groups were analysed using Student's *t*-test, or the nonparametric Mann–Whitney *U*-test for proliferation. *P*-values < 0.05 were considered significant.

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Supplementary material

The following supplementary material is available online:

Fig. S1. Cytoplasmic expression of dipeptidyl peptidase 8 (DP8) and dipeptidyl peptidase 9 (DP9). Transient transfection of the yellow fluorescent protein (YFP) constructs DP8-YFP (A) and DP9-YFP (B) in 293T cells. Fluorescence at 507 nm emission is depicted in green and DAPI derived fluorescence from nuclei is depicted in blue. Confocal images of DP8-green fluorescent protein (GFP) (C) and DP9-GFP (D) expressing AD293 cells show that DP8 had a more diffuse cytoplasmic distribution while DP9 is more localised.

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