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Signaling and Regulation

A Novel Role of Dipeptidyl Peptidase 9 in Epidermal Growth Factor Signaling

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Abstract

Dipeptidyl peptidase IV (DPP4), DPP8, DPP9, and fibroblast activation protein (FAP), the four proteases of the DPP4 gene family, have unique peptidase and extra-enzymatic activities that have been implicated in various diseases including cancers. We report here a novel role of DPP9 in regulating cell survival and proliferation through modulating molecular signaling cascades. Akt (protein kinase B) activation was significantly inhibited by human DPP9 overexpression in human hepatoma cells (HepG2 and Huh7) and human embryonic kidney cells (HEK293T), whereas extracellular signal-regulated kinases (ERK1/2) activity was unaffected, revealing a pathway-specific effect. Interestingly, the inhibitory effect of DPP9 on Akt pathway activation was growth factor dependent. DPP9 overexpression caused apoptosis and significantly less epidermal growth factor (EGF)-mediated Akt activation in HepG2 cells. However, such inhibitory effect was not observed in cells stimulated with other growth factors, including connective tissue growth factor, hepatic growth factor, insulin or platelet-derived growth factor-BB. The effect of DPP9 on Akt did not occur when DPP9 enzyme activity was ablated by either mutagenesis or inhibition. The phosphatidylinositol 3-kinase (PI3K)/Akt pathway is a major downstream effector of Ras. We found that DPP9 and DPP8, but not DPP4 or FAP, associate with H-Ras, a key signal molecule of the EGF receptor signaling pathway. These findings suggest an important signaling role of DPP9 in the regulation of survival and proliferation pathways. *Mol Cancer Res; 9(7); 1–12. ©2011 AACR.*

Introduction

The dipeptidyl peptidase IV (DPP4) gene family consists of 4 atypical serine proteases, including DPP4, fibroblast activation protein (FAP), DPP8, and DPP9. Upon dimerization, this protein family mediates diverse biological processes by releasing the N-terminal dipeptide of substrates with proline at the penultimate position. DPP4 is known to have multiple roles, including T-cell activation and proliferation, metabolic control, and cancer cell biology (1–3), whereas FAP has been implicated in arthritis (4, 5), cancers (6), and lung and liver fibrosis (7–9). DPP8 and DPP9 are ubiquitously expressed in tissues (10, 11). We have previously reported that DPP8 and DPP9 are enriched in epithelial cells, lymphocytes and hepatocytes *in vivo*, and various cancer cell lines, including Jurkat (human T-cell

lymphoma), Raji (human B-cell Burkitt's lymphoma), Huh7 and HepG2 cells (human hepatocellular liver carcinoma cell lines; ref. 10). *In vivo*, DPP9 mRNA expression is significantly induced in testicular cancer (10), whereas DPP8/9 enzyme activity is not significantly up-regulated in gliomas compared to nontumorous human brain (12). The biological functions of DPP8 and DPP9 remain to be elucidated.

Overexpression of DPP9 in human embryonic kidney (HEK293T) cells results in impaired cell adhesion and migration (13), suggesting a role of DPP9 in the regulation of cell growth and mobility. Interestingly, a DPP8/DPP9 selective inhibitor, (2S,3R)-2-(2-amino-3-methyl-1-oxo-pentan-1-yl)-1,3-dihydro-2H-isoindole hydrochloride, attenuates lymphocyte proliferation (14, 15). Recently, the antigenic peptide RUI_{34-42} has been identified as a natural DPP9 substrate, indicating a potential role of DPP9 in cytoplasmic peptide degradation for antigen presentation (16).

Several lines of evidence suggest that DPP4 may be involved in cell signaling. DPP4 overexpression in prostate cancer cells blocks the basic fibroblast growth factor (bFGF) pathway (17). In the Burkitt's B-cell lymphoma line DPP4 overexpression results in elevated p38 mitogen-activated protein kinases [MAPK; extracellular signal regulated kinase (ERK1/2)] phosphorylation (18). Using the nonselective DPP inhibitor Diprotin A, Arscott and colleagues showed that DPP4 enzyme activity downregulates fetal calf serum

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(FCS) stimulated Akt phosphorylation in the neuroblastoma cell line, SK-N-AS (2). However, an important remaining question is whether DPP4 could function as a cytoplasmic signaling enzyme in addition to its extracellular dipeptidyl peptidase activity.

Epidermal growth factor receptor (EGFR) is a prototypical member of the ErbB family of tyrosine kinases that plays important roles in regulation of cell survival, growth, proliferation, and differentiation (19). Upon ligand binding, dimerization of EGFR triggers the activation of its receptor tyrosine kinase activity, resulting in the recruitment and phosphorylation of multiple intracellular substrates and the subsequent transduction of extracellular signals to the nucleus through an intracellular signaling network. EGFR upregulation has been implicated in the pathogenesis of a wide range of human cancers, including breast, brain, colon, lung, and prostate cancers (20). As a result, a number of therapeutic strategies have been developed to target the EGFR signaling axis for the treatment of cancers. Gefitinib and Erlotinib, for instance, are selective EGFR tyrosine kinase inhibitors that have been approved by the US Food and Drug Administration (FDA) for the treatment of advanced non–small cell lung cancer, whereas antibodies Cetuximab and Panitumumab that block ligand binding, and thereby EGFR activation, have been approved for the treatment of metastatic colorectal cancer (21).

One of the well-established downstream signaling pathways of EGFR is the Ras pathway. Activation of EGFR upon ligand binding promotes recruitment of adaptor and signaling molecules that activate Ras. Two of the most important and commonly studied pathways downstream of the H-Ras signaling are the ERK1/2 and the phosphatidylinositol 3-kinase (PI3K)/Akt (protein kinase B) pathways, by which the EGFR regulates various cellular functions, including cell survival, growth, migration, and proliferation (22).

We report here a novel role of DPP9 in attenuating the PI3K/Akt signaling in an EGF-dependent manner, leading to induction of intrinsic apoptosis and suppression of cell proliferation. This is the first indication of the significance of DPP9 enzyme activity in cell signaling.

Materials and Methods

Materials
Antibodies are detailed in Table 1. Other materials were from Sigma-Aldrich unless stated.

Generation of constructs
The DPP9-V5-His and DPP8-V5-His constructs have been described (13). The DPP9-V5-His Ser729Ala enzyme-inactive mutant (mutDPP9-V5-His) was generated by subcloning a 2.4 kb HindIII/PmlI (Eco72I) fragment containing the S729A mutation from DPP9-ECFP S729A (13) into DPP9-V5-His/pcDNA3.1 (11). The DPP8-V5-His Ser739Ala enzyme-inactive mutant (mutDPP8-V5-His) was generated by subcloning a 2.4 kb EcoRI/Xba

Table 1. Antibodies used in immunoblotting and flow cytometry

Antibody	Supplier	Catalog number	Working dilution
Primary antibodies			
Akt (pan; C67E7)	Cell Signaling Technology (Boston, MA)	4691	1000
APC Annexin V	BD Biosciences (Franklin Lakes, NJ)	550474	50
Caspase-3	Cell Signaling Technology (Boston, MA)	9662	1000
Caspase-9	Cell Signaling Technology (Boston, MA)	9502	1000
cH Ras (Y132)	Abcam, Inc. (Cambridge, MA)	ab324117	1000
DPP4 E27	T. Kähne, Magdeburg (26)	–	500
DPP4 B10	T. Kähne, Magdeburg (26)	–	500
DPP8 (TED 1–3)	In-house (10)	–	500
DPP9–Catalytic domain	Abcam, Inc. (Cambridge, MA)	ab42080	5000
FAP F19	Hybridoma supernatant made in-house (24)	ATCC, CRL-2733	3
GAPDH	EnCor Biotechnology (Boulevard, FL)	MCA-1D4	1000
Penta-His Alexa Fluor 488 Conjugate[a]	QIAGEN (Valencia, CA)	35310	200
Phospho-Akt (Ser473; D9E)	Cell Signaling Technology (Boston, MA)	4060	1000
Phospho-ERK (Thr202/Tyr204; E10)	Cell Signaling Technology (Boston, MA)	9106	1000
ERK1/2	Cell Signaling Technology (Boston, MA)	9102	1000
V5[a]	Invitrogen (Carlsbad, CA)	R960-25	200
Secondary antibodies			
Anti-Mouse IgG-HRP	DAKO (Carpinteria, CA)	P0260	3000
Anti-Rabbit IgG-HRP	DAKO (Carpinteria, CA)	P0448	3000
Anti-Mouse IgG-R-PE[a]	Molecular Probes (Invitrogen, Carlsbad, CA)	P852	400

[a]Used for flow cytometry; all other antibodies were used for Western blot.

fragment containing the S739A mutation from DPP8-ECFP S739A (13) into DPP8-V5-His/pcDNA3.1 (11). Transformed, ampicillin-resistant plasmid DNA was purified and completely sequenced. Enzyme activity of the constructs was measured on 50 μg lysates of transfected cells using the fluorescent substrate H-Ala-Pro-AFC as described (10).

Cell culture

HepG2 and Huh7 cells were transiently transfected with wtDPP9-V5-His, mutDPP9-V5-His, wtDPP8-V5-His, mutDPP8-V5-His, or vector control (pcDNA3.1/V5-HisA; Invitrogen) in complete DMEM supplemented with 10% FCS. HepG2 and Huh7 cells were obtained from the American Type Culture Collection annually. For detection of growth factor stimulated phosphorylation, cells at 24 hours posttransfection were cultured in serum-free DMEM overnight to suppress basal phosphorylation signals. Forty hours posttransfection, cells were stimulated with various growth factors, including CTGF (ref. 23; 0, 25, 250 ng/mL, 15 minutes), EGF (0, 10, 15 ng/mL, 15 to 30 minutes; catalog number 236-EG, R&D Systems), HGF (0, 10 ng/mL, 10 minutes; catalog number 294-HG-005, R&D Systems), insulin (0, 10, 100 nM, 30 minutes; catalog number 12643, Sigma-Aldrich), and PDGF-BB (0, 20, 50 ng/mL, 10 minutes; catalog number GF149, Chemicon, Temecula). Some HepG2 cells maintained in complete DMEM were serum starved overnight to suppress phosphorylation signals followed by treatment for 1 hour with 0 or 5 μM DPP8/9 inhibitor (DPP8/9i), (2S,3R)-2-(2-amino-3-methyl-1-oxopentan-1-yl)-1,3-dihydro-2H-isoindole hydrochloride (2:1; kind gift from Professors Pieter Van der Veken and Koen Augustyns, University of Antwerp), before stimulation with 10 ng/mL EGF for 15 minutes at 37°C. The diluent control for DPP8/9i was 0.5% methylcellulose. Some HepG2 cells maintained in complete DMEM were serum starved overnight then incubated with 0, 5, or 50 μM PI3K inhibitor LY294002 (catalog number L9908, Sigma-Aldrich) for 1 hour before stimulation with 20 ng/mL EGF for 15 minutes at 37°C. The diluent control for LY294002 was 0.5% DMSO.

Immunoblotting assays

Cells were washed with ice-cold PBS 3 times before harvest in ice-cold lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 1 mM MgCl₂, 150 mM NaCl, 1× Roche complete protease inhibitor cocktail (Roche Applied Science), 10 mM sodium fluoride, 0.25% sodium deoxycholate, 100 μM sodium-orthovanadate, 1% Triton-114, 10% glycerol, pH 7.6) and stored at −80°C. Human liver tissues were obtained from liver transplant recipients in accordance with National Health and Medical Research Council guidelines under Royal Prince Alfred Hospital Human Ethics Committee approvals. Nondiseased liver donors had an age range of 6 to 58 and mixed genders. Cirrhotic livers were from primary biliary cirrhosis (PBC) patients of average age 51.7 ± 13.3 (range 27–67; 10 females, 2 males) and end stage alcoholic liver disease (ALD) patients of average age 49.3 ±

8 (range 34–60, 9 males). Protein concentration was determined using the Micro BCA Protein Assay Kit (catalog number 23235, Thermo Scientific) following the manufacturer's protocol. Fifty micrograms of total cell lysates or 80 μg tissue lysates were resolved on 4% to 12% Bis-Tris SDS-PAGE (catalog number NP0323BOX, Invitrogen) or 3% to 8% Tris-Acetate SDS-PAGE (catalog number EA03755BOX, Invitrogen) followed by immunoblotting. Relative band intensities were quantitatively analyzed using Image J and normalized against control proteins as indicated.

Apoptosis assay

Transfected cells were serum-starved for 4 hours prior to treatment with 0, 5, or 50 μM LY294002 for an hour then 20 ng/mL EGF overnight. Cells were harvested by trypsinization then washed with annexin binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂). Staining involved incubating cells with APC annexin V (diluted 1:50, catalog number 550474, BD Biosciences) for 30 minutes at RT in the dark followed by PI at 50 μg/mL in PBS (Sigma-Aldrich catalog number P4170) for 5 minutes. Annexin V positive cells that were PI-negative were considered to be the apoptotic cells. Other procedures have been described (24).

MTS proliferation assay

Transfected cells were seeded onto 96-well plates at 3,000 cells per well in serum free media for 4 hours before treatment. MTS assay was performed using the CellTiter 96 AQ_ueous Non-Radioactive Cell Proliferation Assay kit (catalog number G5421, Promega). Absorbance was measured at 490 nm every 24 hours after the initial reading.

Cell cycle analysis

Transfected HepG2 cells at 10^6/mL were fixed in 4% formaldehyde for 10 minutes, washed with 5% FCS, 0.01% sodium azide, 2 mM EDTA, 0.5% Saponin in PBS, then stained for 30 minutes at room temperature with antibodies at 1:200 to the V5 or polyhistidine tag. Following another wash, cells were incubated with freshly made DAPI staining solution (2 mg/mL DAPI in PBS) for 30 minutes at 37°C. Cell analysis used a BD LSR II flow cytometer.

Coimmunoprecipitation

Five hundred micrograms of total HeLa (human cervix carcinoma; ATCC number CCL2) cell lysate was precleared 3 times with 250 ng rabbit immunoglobulin G (IgG; Invitrogen) and 25 μL Protein A Sepharose 4B (Amersham Pharmacia) as described (25). Precleared lysate was incubated with 1 μg rabbit anti-H-Ras MAb (catalog number ab324117; Abcam) for 90 minutes. The antibody–antigen complex was captured by 20 μL Protein A Sepharose 4B. The immuno-agarose complex was washed by brief centrifugation 5 times with ice-cold PBS containing 0.1% Triton-X114. All procedures were performed at 4°C in the presence of protease inhibitor cocktail. The immunocomplex was resuspended in 200 μL TE buffer (pH 7.6) and 50 μL of this suspension was incubated with

H-Ala-Pro-AFC to measure DPP4 enzyme activity (11). For immunoblotting, the immunocomplex was heated in 25 μL sample buffer (20% glycerol, 6% SDS, 10% 2-mercaptoethanol) at 90°C for 10 minutes before being subjected to 3% to 8% Tris-acetate SDS-PAGE and subsequent immunoblotting.

Results

DPP9 overexpression induces intrinsic apoptosis in HepG2 cells

We had previously showed that overexpression of DPP9 induces spontaneous apoptosis in HEK293T cells in the absence of external stimuli, suggesting a role of DPP9 in regulating the intrinsic apoptotic pathway (13). Therefore, we investigated whether DPP9 enzyme activity induces intrinsic apoptosis by measuring the extent of caspase 9 activation. Cleavage of caspase 9 has served as a hallmark of intrinsic apoptosis. The levels of such active forms of caspase 9 (18, 35, and 38 kDa) and caspase 3 (15 and 20 kDa) were significantly elevated in cells transfected with wtDPP9 compared to an enzyme negative Ser729AlaDPP9 mutant, which lacked DPP activity due to mutation of the catalytic serine (Fig. 1). Natural bovine DPP9 has a gel mobility at approximately 100 kDa (26). Endogenous DPP9 in HepG2 cells was detected by immunoblot as 2 bands, at approximately 95 and 110 kDa, whereas the recombinant DPP9-V5-His had an electrophoretic mobility of about 97 kDa (Figs. 1D and 2A).

DPP9 attenuates activation of Akt, but not ERK

The signaling pathways p42/p44 ERK1/2 and PI3K/Akt cascades are central regulators for cell survival. Therefore, we investigated the effect of DPP9 on the ERK1/2 and PI3K/Akt pathway activation. Both Akt and ERK1/2 were activated upon FCS stimulation in a dose-dependent manner.
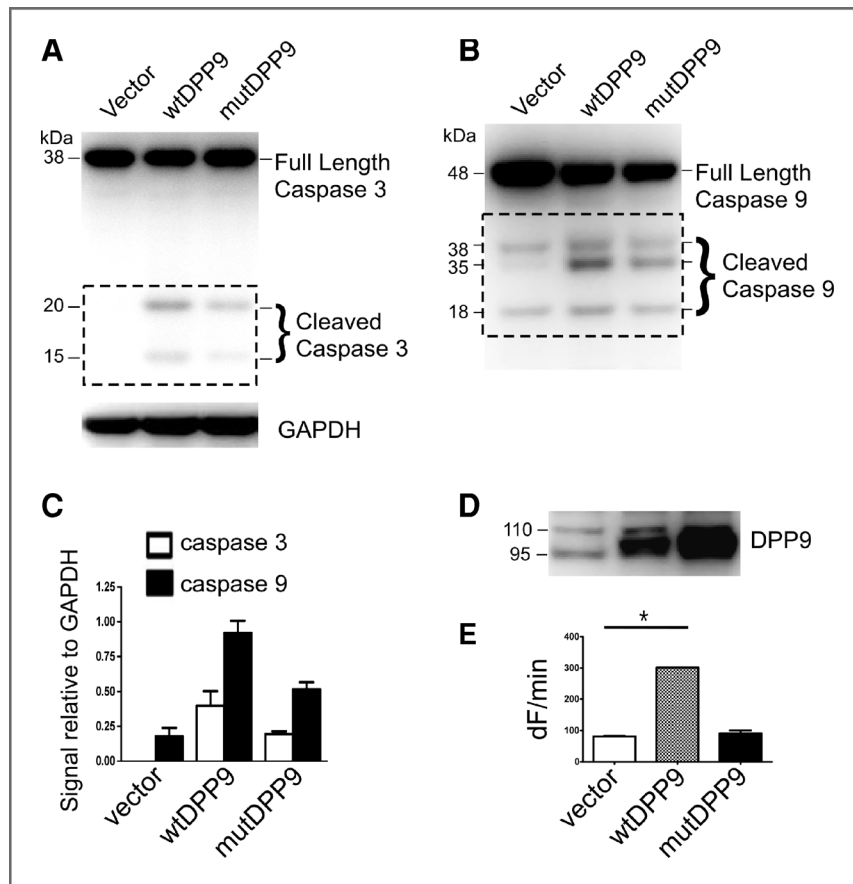

Figure 1. DPP9 induced intrinsic apoptosis in HepG2 cells, measured by activated caspases 3 and 9. Vector, wtDPP9-V5-His, or mutDPP9-V5-His transfected HepG2 cells were serum starved for 48 hours then assessed for caspase 3 (A) and 9 (B) activation. Increased levels of activated caspases 3 and 9 (boxed) were observed in HepG2 cells overexpressing wtDPP9-V5-His compared to vector or mutDPP9-V5-His. Representative of 2 experiments. C, densitometry analyses of caspase 3 and 9 in A and B normalized against GAPDH. D, DPP9 overexpression was shown by immunoblotting the same gels. Endogenous DPP9 was detected at approximately 95 and 110 kDa, whereas the recombinant DPP9-V5-His was detected at approximately 97 kDa. (E) DPP9-V5-His mutated at the catalytic serine (Ser729Ala) lacked DPP-IV (H-Ala-Pro-AFC) activity: 50 μg of total cell lysate from vector, wt, or mutDPP9-V5-His transfected HepG2 cells was assayed for DPP activity using the DPP-IV substrate H-Ala-Pro-AFC. Enzyme activity units are the average change in arbitrary fluorescence units per minute (dF/min). *, $P < 0.001$ compared to the wtDPP9-V5-His. Representative of 3 independent transfections.
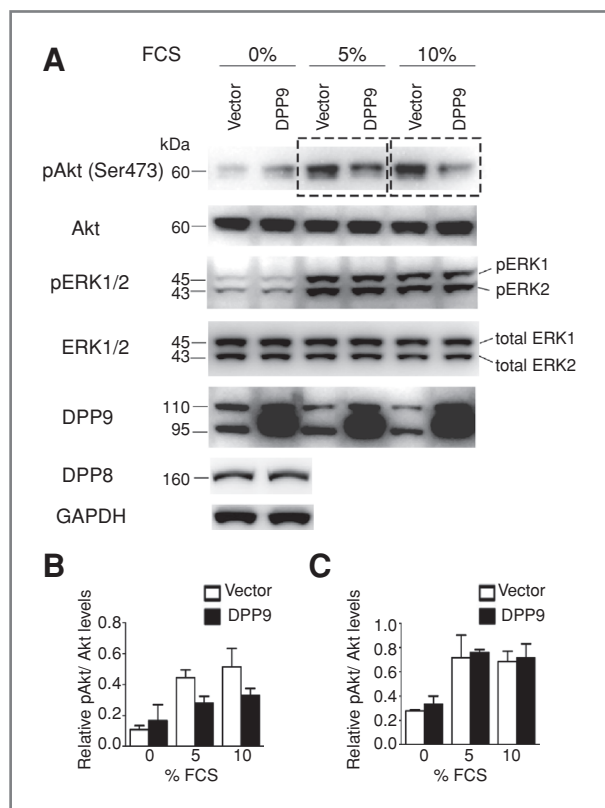
Figure 2. DPP9 attenuated Akt, but not ERK1/2 phosphorylation, in HepG2 cells stimulated with FCS. The extent of Akt and ERK1/2 phosphorylation is an indication of pathway activation. A, vector or DPP9-V5-His transfected HepG2 cells were serum starved for 16 hours before stimulation with 0%, 5%, or 10% FCS for 30 minutes at 37°C. Cell lysates were analyzed by immunoblot using the antibodies indicated at the left. With 5% FCS and 10% FCS stimulation, the phospho-Akt (pAkt), but not the phospho-ERK1/2 (pERK1/2), signal was weaker in cells transfected with DPP9-V5-His compared to vector control (boxed). DPP9 overexpression was comparable in each DPP9 transfection and did not alter DPP8 expression intensity. Densitometry analyses of the relative phosphoAkt and phosphoERK1/2 levels are shown in B and C, respectively. Representative of 2 experiments.

Remarkably, overexpression of DPP9 in HepG2 cells (Fig. 2) and 293T (Supplementary Fig. S1) down-regulated the phosphorylation of Akt, whereas total Akt expression levels were not changed compared to vector control, indicating an inhibitory effect of DPP9 on Akt activation. By contrast, neither phosphorylated ERK1/2 nor total ERK1/2 were influenced by DPP9 overexpression, suggesting a specific effect of DPP9 on the Akt pathway. Endogenous DPP8 expression, which in some cell lines is detected at 160 kDa on SDS-PAGE (Fig. 2A; data not shown), was unaffected by DPP9 overexpression (Fig. 2), so DPP8 probably does not contribute to the observed Akt inhibition.

The inhibitory effect of DPP9 on Akt phosphorylation is EGF specific

Having documented the effect of DPP9 on FCS-induced Akt activation, the influence of DPP9 on growth factor

signaling was therefore investigated. No significant differences in the extent of Akt and ERK1/2 phosphorylation were observed between vector and DPP9 transfected HepG2 cells when stimulated with CTGF, HGF, insulin, or PDGF at a range of concentrations (Fig. 3). Interestingly, HepG2 cells exhibited less Akt phosphorylation in response to EGF stimulation when DPP9-transfected (Fig. 4). The time-course analysis shows that the DPP9-mediated inhibition was sustained over the period of Akt activation (5–30 minutes post-EGF stimulation), suggesting that DPP9-mediated inhibition affects the process rather than the kinetics of Akt activation.

The inhibitory effect of DPP9 on Akt depends on DPP9 enzymatic activity

Because DPP9 enzyme activity is required for induction of intrinsic apoptosis, we tested whether DPP9 enzyme activity is required for inhibition of Akt phosphorylation. Interestingly, the enzyme negative Ser729Ala mutDPP9 failed to attenuate Akt phosphorylation in both HepG2 and Huh7 cell lines (Fig. 5A–D), indicating that the inhibitory effect of DPP9 on Akt is dependent on its DPP activity and that it is not cell line specific. EGF stimulation did not alter the level of DPP9 enzyme activity (Supplementary Fig. S2). Although highly homologous to DPP9, DPP8 enzyme activity did not affect Akt activation (Fig. 5E and F). Inhibition of DPP9 enzyme activity using an inhibitor of DPP8 and DPP9 provided further evidence that the DPP9-mediated downregulation of Akt phosphorylation was primarily due to DPP9 enzyme activity (Fig. 5G–I).

Modulation of mitogenic signaling by DPP9

Because the Akt pathway provides crucial antiapoptotic and prosurvival signals, we investigated whether inhibition of Akt activation by DPP9 enzyme activity is capable of blocking the PI3K/Akt mediated antiapoptotic effect. In agreement with our previous report (13), overexpression of wtDPP9 in HepG2 cells resulted in a significant increase in apoptosis. Notably, the wtDPP9-transfected cells exhibited more cell death than the mutant-transfected cells (Fig. 6A and Supplementary Fig. S3). Similarly, in the presence of EGF significantly greater percentages of apoptotic cells were observed in wtDPP9 (2%–3%) compared to mutDPP9 (0.6%–1.1%) transfected cells (Fig. 6A). By contrast, EGF did not alter the extent of increased apoptosis conferred by DPP9 enzyme activity, suggesting an EGF-independent mechanism whereby DPP9 induces apoptosis. Serving as a control, the PI3K inhibitor (LY294002) treated cells showed significantly more apoptosis either with or without EGF stimulation (Fig. 6B and Supplementary Fig. S4).

Akt signaling is known to regulate cell proliferation, therefore we examined whether inhibition of Akt signaling by DPP9 alters cell proliferation, determined by the MTS assay. EGF promoted proliferation of HepG2 cells (Fig. 7). Interestingly, wtDPP9 overexpressing cells showed about 50% less MTS activity at time 48 and 72 hours post-replating compared to mutDPP9- or vector-transfected cells in the presence of EGF, suggesting that DPP9 enzyme activity may be
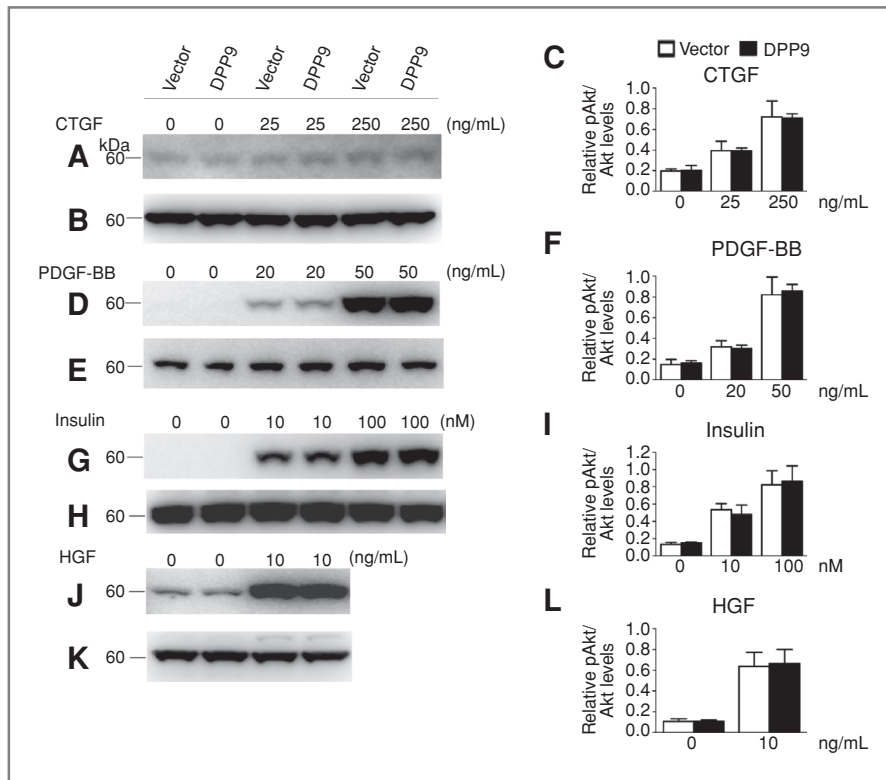
Figure 3. DPP9 overexpression did not downregulate Akt phosphorylation induced by CTGF, PDGF-BB, insulin, or HGF. Vector or DPP9-V5-His transfected HepG2 cells were serum starved for 16 hours before stimulation with CTGF for 15 minutes, PDGF-BB for 10 minutes, insulin for 30 minutes, or HGF for 10 minutes at the indicated concentrations. Cell lysates were analyzed by immunoblot using antibodies to phospho-Akt (pAkt; A, D, G, J) and total Akt (B, E, H, K). Densitometry analyses of the relative phosphoAkt to total Akt levels for each growth factor stimulation are shown to the right (C, F, I, L). Representative of 2 experiments.

involved in attenuating EGF-mediated HepG2 cell proliferation. Notably, this effect was similar without EGF treatment, suggesting an anti-proliferative role of DPP9 enzyme activity independent of EGF signaling. Consistent with the DPP9 data, LY294002 treated cells showed a significant decrease in MTS activity compared to untreated cells, indicating that the

PI3K/Akt pathway is important for proliferation of these cells (Fig. 7C). Although DPP4 and FAP have been documented to cause cell cycle arrest (17, 27) and cell cycle arrest was anticipated from the proliferation data, we did not detect any influence of DPP9 on cell cycle in the conditions of our assay (Supplementary Fig. S3C).
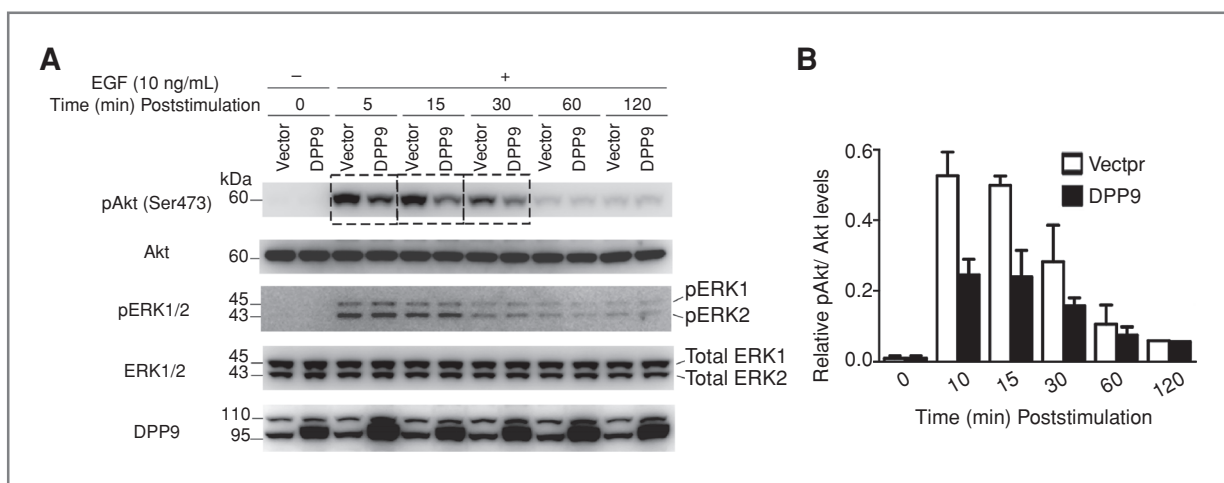

Figure 4. DPP9 inhibited EGF-induced Akt phosphorylation in a sustained manner. A, vector or DPP9-V5-His transfected HepG2 cells were serum starved for 16 hours then stimulated with 10 ng/mL of EGF at 37°C for 0 to 120 minutes. Immunoblots used the antibodies indicated. Akt activation was down-regulated in DPP9 transfected cells in a sustained fashion over the period of Akt phosphorylation (boxed). In contrast, phospho-ERK1/2 was unaffected. The Akt and ERK1/2 immunoblots were included as controls. The DPP9 immunoblot shows that DPP9 overexpression was comparable in each DPP9 transfection. B, densitometry analyses of phosphoAkt levels normalized to total Akt. Representative of 2 experiments.
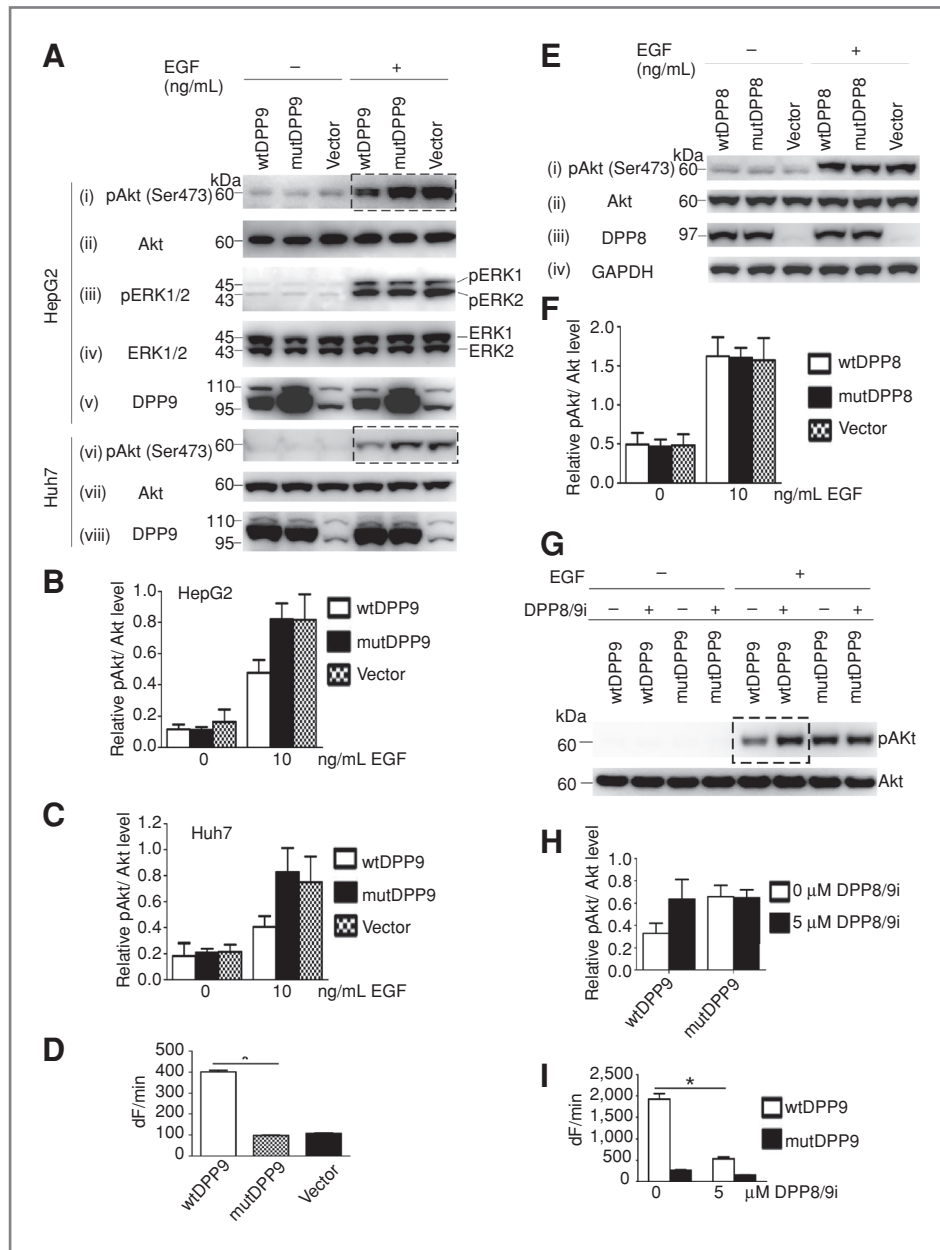
Figure 5. The attenuating effect of DPP9 on Akt phosphorylation was dependent upon DPP9 enzyme activity. A, HepG2 cells (i–v) or Huh7 cells (vi–viii) transiently transfected with vector, wild-type, or enzyme inactive mutDPP9-V5-His (Ser729Ala) were serum starved for 16 hours then treated with EGF at 0 or 10 ng/mL for 15 minutes at 37°C. Cell lysates were analyzed by immunoblot using the antibodies indicated. WtDPP9-V5-His transfected cells showed less Akt phosphorylation compared to mutDPP9-V5-His or vector transfected cells (boxed). In contrast, phospho-ERK1/2 was unaffected. B, densitometry analyses of relative phospho-Akt (i) levels normalized against total Akt (i, ii). C, densitometry analyses of relative phospho-Akt (vi) levels normalized against total Akt (vii). D, DPP9-V5-His mutated at the catalytic serine (Ser739Ala) lacked DPP-IV activity (H-Ala-Pro-AFC). E, neither DPP8 nor its enzyme activity affected Akt phosphorylation. F, densitometry E immunoblots showing phospho-Akt relative to total Akt (Ei, Eii); there was no significant difference in phosphoAkt levels between wtDPP8 and mutDPP8 transfected cells (*$P > 0.05$). G, HepG2 cells transiently transfected with wild-type or enzyme inactive mutDPP9-V5-His (Ser729Ala) were serum starved for 16 hours before treatment with 0 or 5 μM DPP8/9 inhibitor (DPP8/9i), for 20 minutes. This was followed by stimulation with EGF at 0 or 10 ng/mL for 15 minutes at 37°C. In wtDPP9 overexpressing cells, less Akt phosphorylation was observed in vehicle, but not DPP8/9i, treated cells. H, densitometry analyses of the relative phospho-Akt levels in G normalized against total Akt. I, 5 μM of DPP8/9i inhibited >75% of the DPP-IV activity of DPP9-transfected HepG2 cells. Transfection levels were high and comparable for wt-DPP9, mut-DPP9, wt-DPP8, and mutDPP8 (Supplementary Fig. S3). Representative of 2 experiments.

In vitro binding of DPP9 to H-Ras

One of the key regulators of Akt activation is H-Ras. We investigated the potential for interactions between H-Ras and DPP9 in the HeLa cell line, which was chosen due to high levels of endogenous H-Ras and DPP9 expression. The association of H-Ras and DPP9 was shown by
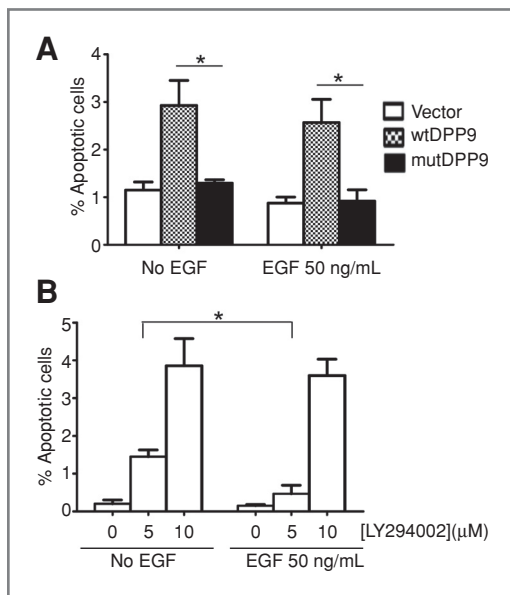
Figure 6. DPP9 enzyme activity up-regulated apoptosis in HepG2 cells in the presence of EGF (A), similar to the effect of LY294002 (B), which blocks the PI3K/Akt signaling pathway. A, vector, wtDPP9-V5-His, or enzyme-negative mutDPP9-V5-His transfected HepG2 cells were serum starved for 4 hours then treated with EGF (50 ng/mL) for 24 hours. B, HepG2 cells serum starved for 16 hours were treated with 0, 5, or 50 μM LY294002 for an hour before EGF treatment (50 ng/mL) for 24 hours. Annexin V positive/PI negative apoptotic cells were enumerated using flow cytometry. Mean ± SD from 3 independent transfections. *, $P < 0.001$ compared to the wtDPP9-V5-His.

Figure 7. DPP9 enzyme activity down-regulated HepG2 cell proliferation (A, B), similar to the effect of the PI3K inhibitor LY294002 (C), which blocks the PI3K/Akt signaling pathway. Vector, wtDPP9, or enzyme-negative mutDPP9 transfected HepG2 cells were replated in serum-free media for 4 hours before stimulation with 0 or 20 ng/mL EGF (A, B). C, HepG2 cells serum starved for 4 hours were treated with 0, 5, or 50 μM LY294002 for an hour before stimulation with 0 or 20 ng/mL EGF. Cell proliferation (A490 nm) was determined by MTS assay. Mean ± SD from 3 independent transfections (*$P < 0.01$).

coimmunoprecipitation of endogenous H-Ras and its ligands. H-Ras associated proteins that were pulled down by antibodies against H-Ras exhibited DPP activity measured by hydrolysis of the DPP substrate H-Ala-Pro-AFC (Table 2). This data is concordant with immunoblots of the immunocomplexes of H-Ras, which included DPP8 and DPP9, but not DPP4 or FAP (Fig. 8). This probably reflects structural features: DPP8 and DPP9 are structurally similar (79% amino acid similarity and 61% amino acid identity), whereas DPP4 and FAP are highly homologous (52% amino acid identity), but only 27% identical to DPP8 and DPP9 (11). DPP9 was detected as a strong 80 kDa and a weaker 130 kDa band, whereas DPP8 was seen at approximately 110 and 150 kDa with similar band intensities. The DPP4 dimer runs unexpectedly fast at about 150 kDa from a 110 kDa monomer (25, 28), so DPP9 at 130 kDa and DPP8 at 150 kDa may be dimers whereas the faster bands are monomer and possibly fragments (10, 11).

DPP9 upregulation in cirrhotic human livers

DPP9 is expressed in hepatocytes and lymphocytes (10). Compared to nondiseased liver, lymphocytes are more numerous in cirrhotic liver and hepatocytes of cirrhotic liver often progress to hepatocellular carcinoma. It is therefore interesting to compare the expression of intrahepatic DPP9 in nondiseased and cirrhotic livers. DPP9 immuno-
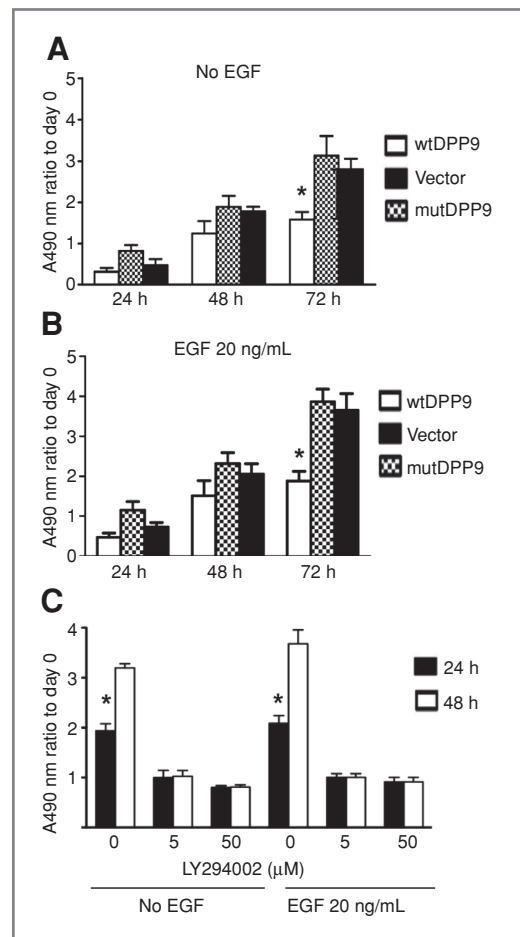
blots on liver samples of 2 cirrhotic etiologies, alcoholic, and biliary, showed DPP9 downregulation in end stage chronic human liver injury. Both ALD and PBC livers had approximately 30% less DPP9 expression, detected at 90 kDa (Fig. 9).

Discussion

This is the first report of a protein ligand of DPP8 and DPP9 and of a role for DPP9 enzyme activity in cell signaling. We showed that DPP9 and its homolog DPP8 associate with the oncogene H-Ras. We also showed a novel biological significance of DPP9 enzyme activity in EGF-mediated signaling and cellular function. Our data indicate important roles of DPP9 enzyme activity in mitogenic signaling, and thus of cell functions including apoptosis

Table 2. Coimmunoprecipitation of dipeptidyl peptidase activity with H-Ras from HeLa cells

Immunoprecipitation experimental design	Activity	
	Mean (SD)	*P*
Anti-H-Ras + lysate	4.25 (0.14)	<0.001
No antibody + lysate	2.33 (0.42)	
No-lysate control	2.45 (0.25)	

and proliferation that are inadequately regulated in most cancers. We showed that DPP9 enzyme activity attenuates PI3K/Akt, but not ERK1/2 pathway activation. Among the 5 growth factors examined, including CTGF, EGF, HGF, insulin, and PDGF, EGF was the only growth factor that exhibited DPP9-mediated inhibition of Akt phosphorylation in HepG2 and Huh7 cells. Of interest for the pathogenesis of liver disease, we showed that DPP9 is downregulated in liver cirrhosis.

It has been reported that DPP4 enzyme activity suppresses FCS-stimulated Akt phosphorylation in SK-N-AS cells (2). In that study, introduction of the nonselective DPP inhibitor Diprotin A (the tripeptide Ile-Pro-Ile) to DPP4 transfected cells blocked the DPP4-mediated antiphospho Akt effect (2). As Diprotin A inhibits all peptidases of the DPP4 family, its effect on Akt activation might be due to inhibition of DPP8 and/or DPP9, rather than DPP4 *per se*. A DPP9-selective inhibitor is not available so an inhibitor of both DPP8 and DPP9 was used to show involvement of DPP9 enzyme activity. Specificity for DPP9 was indicated in this study by showing that EGF-stimulated Akt phosphorylation was unaffected in DPP8 overexpressing cells. Whether the effect of DPP4 on Akt is ligand specific has not been established. Differences in cellular localization between DPP4 and DPP9 suggest that these 2 peptidases might attenuate Akt activation through distinct mechanisms. DPP4 is mainly expressed on the cell surface, whereas DPP9 expression is exclusively intracellular (11). Nevertheless, a phosphorylated form of DPP4 has been reported in cytoplasm (29).

The PI3K/Akt pathway has a crucial role in maintaining cell survival by protecting the cell from intrinsic apoptosis (22). Consistent with the inhibitory effect of DPP9 on Akt activation, cells overexpressing wtDPP9 showed significantly more apoptosis compared to enzyme negative mutDPP9 transfected cells under EGF stimulation, indicating that DPP9 enzyme activity may regulate the protective effect of EGF against apoptosis. Nevertheless, these data might alternatively suggest an EGF-independent mechanism that is yet to be characterized whereby DPP9 enzyme activity induces apoptosis. Consistent with PI3K/Akt pathway inhibition, we showed that DPP9 enzyme activity curtails proliferation and induces intrinsic apoptosis in HepG2 cells.
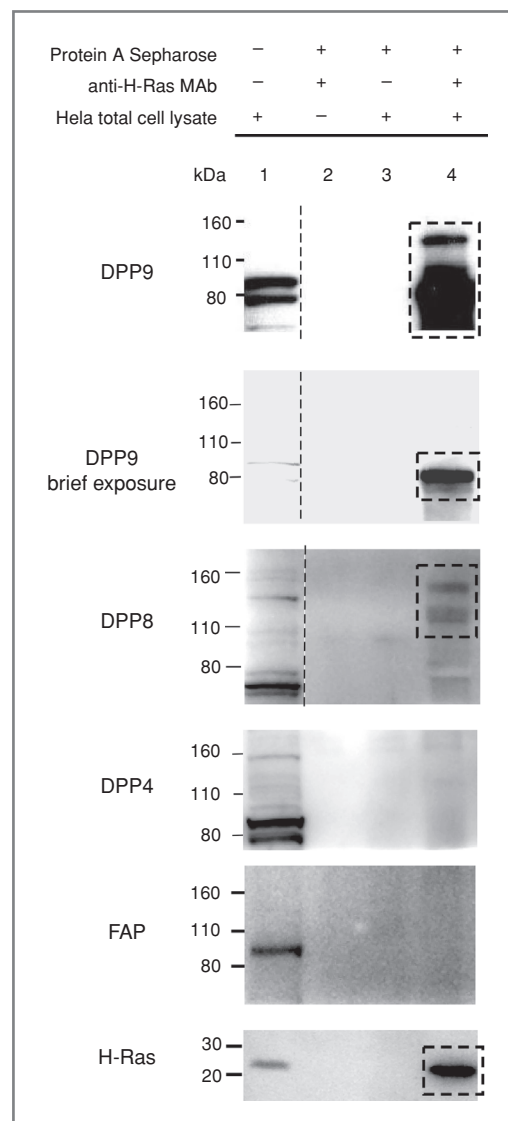

Figure 8. Coimmunoprecipitation of endogenous H-Ras, DPP8, and DPP9 from HeLa total cell lysate using anti-H-Ras MAb. The coimmunoprecipitation products were immunoblotted with antibodies to DPP9, DPP8, FAP, DPP4, or H-Ras as indicated. Lane 1: precleared HeLa total cell lysate. Lanes 2 to 4: protein A Sepharose following the indicated incubations. Samples were reduced and boiled except for the anti-FAP immunoblot, which was performed on nonreduced/nonboiled samples. Both DPP8 and DPP9 were coimmunoprecipitated with H-Ras (boxed). The forms of DPP8 that associated with H-Ras appeared to differ in electrophoretic mobility from those that were most readily detected in the precleared HeLa total cell lysate. The vertical dashed lines indicate nonadjacent gel lanes. Representative of 3 experiments.

DPP9 may have a role in cancer cell development through influencing the PI3K/Akt signaling pathway. DPP9 mRNA levels are elevated in testicular tumors (10), which have a deregulated increase in Akt activation (30), and DPP9 is most readily detected in transformed cells (11). Perhaps upregulation of DPP9 in tumors serves as a mechanism to dampen the progression of cancer cell growth, proliferation, and survival.
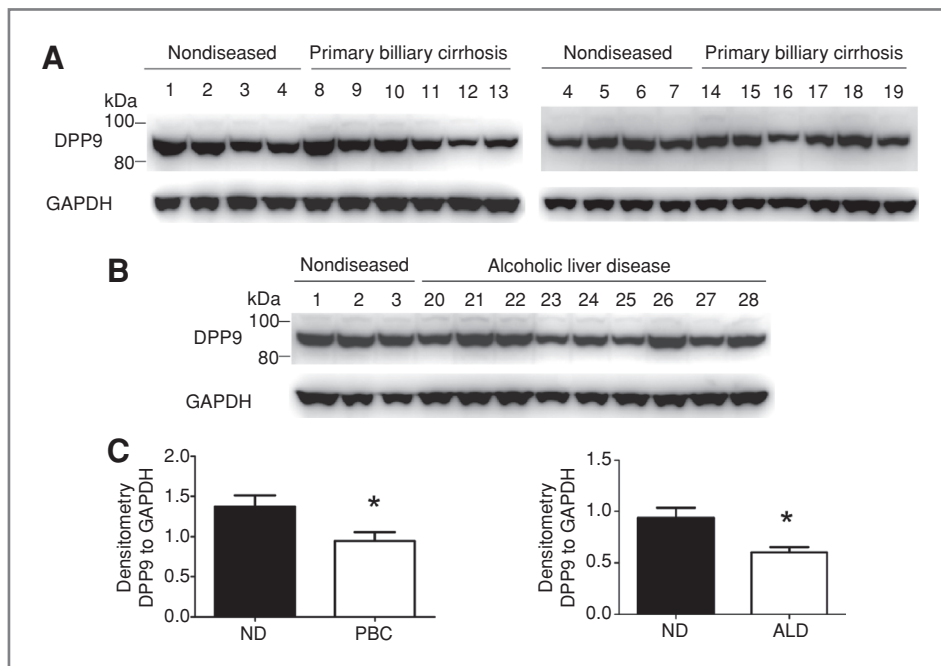
Figure 9. DPP9 protein expression downregulation in cirrhotic human liver. Immunoblots with anti-DPP9 on nondiseased liver (7 patients, lanes 1–7) and on cirrhotic liver from primary biliary cirrhosis (12 patients, lanes 8–19) and alcoholic liver disease (9 patients, lanes 20–28) patients (A, B). DPP9 had a mobility of approximately 95 kDa in these conditions. GAPDH was the loading control. C, densitometry data of DPP9 proportional to GAPDH (mean ± SD). *, $P < 0.05$.

DPP9 is expressed in lymphocytes and epithelial cells, notably hepatocytes *in vivo* and hepatocarcinoma cell lines *in vitro* (10, 11). DPP9 protein was down-regulated in ALD and PBC cirrhotic human livers compared to nondiseased controls. These diseases often progress to hepatocellular carcinoma. DPP9 is redox sensitive (31), so DPP9 enzyme activity might be further down-regulated by the increased oxidation that occurs in damaged hepatocytes. This DPP9 down-regulation in cirrhosis occurs at a time of EGF signaling upregulation (32), and may contribute to increased Akt activation that has been detected in cirrhotic human livers (33). Perhaps diminished DPP9 expression enhances growth factor signaling and/or lessens apoptosis, and thus contributes to increased hepatic cell proliferation in end-stage liver diseases, facilitating progression to hepatocellular carcinoma.

Redox regulation of DPP9 might be involved in modulating the PI3K/Akt pathway. We have shown that DPP9 enzyme activity is reversibly inactivated by oxidation (31). An oxidative cellular environment created under oxidative stress or production of reactive oxygen species during cellular metabolism, such as growth factor stimulation, inhibits the PI3K/Akt pathway through inactivation of PTEN, a PtdIns(3,4,5)P3 phosphatase that antagonizes PI3K function (34). As the enzyme activities of PTEN and DPP9 are both redox state dependent, the redox condition might be a molecular switch in PI3K/Akt pathway regulation.

Because DPP9 is a cytoplasmic protein, endosomal signaling of EGFR is a potential route for DPP9 to modulate Akt activation. Activation of H-Ras by EGF/EGFR within endosomes has been reported by several groups (35–37). In human epidermal carcinoma A-431 cells, endosomal EGFR/H-Ras complexes are formed by fusion of endosomes that contain EGFR with endosomes that contain H-Ras (35). Endosomal EGFR signaling alone is sufficient to activate the major signaling pathways, including the Akt cascade that regulates cell proliferation and survival (38). A preferential enrichment of H-Ras over K-Ras in endosomes can occur in A-431 cells (39). Further investigation might determine whether DPP9 is recruited to the endosomal membrane in a complex with H-Ras upon EGFR activation, where DPP9 may exert its attenuating effect toward Akt phosphorylation, perhaps by cleaving one or more signaling or scaffolding molecules.

In summary, this study is the first indication of the significance of DPP9 enzyme activity in regulating cell behaviors, and the first data on a role of DPP9 in cell signaling. We showed that DPP9 enzyme activity attenuates PI3K/Akt signaling in a growth factor specific manner, resulting in augmented apoptosis and suppressed cell proliferation. The effect of DPP9 on the PI3K/Akt pathway activation will need to be examined *in vivo*. It would be interesting to identify specific DPP9 substrates involved in attenuating the EGFR/PI3K/Akt pathway, which would provide a greater understanding of EGF action and Akt signaling.

Disclosure of Potential Conflicts of Interest

Acknowledgments

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