Protein-tyrosine Phosphatase PCP-2 Inhibits β-Catenin Signaling and Increases E-cadherin-dependent Cell Adhesion*

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He-Xin Yan†, Wen Yang‡, Rui Zhang†, Lei Chen†, Liang Tang†, Bo Zhai†, Shu-Qin Liu†, Hui-Fang Cao†, Xiao-Bo Man†, Hong-Ping Wu†, and Meng-Chao Wu†, and Hong-Yang Wang†‡§

From the †International Cooperation Laboratory on Signal Transduction, Eastern Hepatobiliary Surgery Institute, Second Military Medical University, Shanghai 200438 and the ‡National Laboratory for Oncogene and Related Genes, Cancer Institute of Shanghai Jiao Tong University, Shanghai 200032, China

β-Catenin is a key molecule involved in both cell adhesion and Wnt signaling pathway. However, the exact relationship between these two roles has not been clearly elucidated. Tyrosine phosphorylation of β-catenin was shown to decrease its binding to E-cadherin, leading to decreased cell adhesion and increased β-catenin signaling. We have previously shown that receptor-like protein-tyrosine phosphatase PCP-2 localizes to the adherens junctions and directly binds and dephosphorylates β-catenin, suggesting that PCP-2 might regulate the balance between signaling and adhesive β-catenin. Here we demonstrate that PCP-2 can inhibit both the wild-type and constitutively active forms of β-catenin in activating target genes such as c-myc. The phosphatase activity of PCP-2 is required for this effect since loss of catalytic activity attenuates its inhibitory effect on β-catenin activation. Expression of PCP-2 in SW480 colon cancer cells can lead to stabilization of cytosolic pools of β-catenin perhaps, by virtue of their physical interaction. PCP-2 expression also leads to increased membrane-bound E-cadherin and greater stabilization of adherens junctions by dephosphorylation of β-catenin, which could further sequester cytosolic β-catenin and thus inhibit β-catenin mediated nuclear signaling. Furthermore, SW480 cells stably expressing PCP-2 have a reduced ability to proliferate and migrate. Thus, PCP-2 may play an important role in the maintenance of epithelial integrity, and a loss of its regulatory function may be an alternative mechanism for activating β-catenin signaling.

Reversible and dynamic tyrosine phosphorylation is controlled by the opposing actions of protein-tyrosine kinases and protein-tyrosine phosphatases (PTPs)1 (1). PTPs are a large family that is broadly classified into receptor-like protein-tyrosine phosphatases (RPTPs) and cytosolic PTPs (2). A subfamily of RPTPs containing an MAM (Meprin/A5/PTP into receptor-like protein-tyrosine phosphatases (RPTPs) and cytosolic signaling.

1 Address correspondence to: Hong-Yang Wang, M.D. International Cooperation Laboratory on Signal Transduction, Eastern Hepatobiliary Surgery Institute, Second Military Medical University, 225 Changhaid Road, 200438 Shanghai, China. Tel: 86 21 2507 0856; Fax: 86 21 6556 6851; E-mail: hywangk@vip.sina.com.

2 The abbreviations used are: PTP, protein-tyrosine phosphatases; RPTP, receptor-like PTP; MAM-PTP, MAM-subfamily PTP; APC, adenomatous polyposis coli; EGF, epidermal growth factor; EGFR, EGF receptor; PBS, phosphate-buffered saline; siRNA, small interfering RNA; Tcf, T cell factor; ChIP, chromatin immunoprecipitation; WT, wild type; mu, mutant.

...fibronectin type III repeats is defined as the MAM-subfamily PTPs (MAM-PTPs), which include PCP-2, PTPμ, PTPκ, and PTPπ (3, 4). These RPTPs contain a single membrane-spanning region with two cytoplasmic PTP domains. The intracellular juxtamembrane domain of these RPTPs contains a region that is homologous to the conserved intracellular domain of the cadherins (5).

Cadherins are a family of calcium-dependent adhesion molecules that play an essential role in the formation of the cell-cell contacts termed adherens junctions. Cadherin-mediated cell-cell adhesion is important for development and maintenance of epithelial tissue integrity, and its disturbance contributes to the invasive and metastatic phenotype of epithelial tumors. Through their intracellular domains, cadherins associate with molecules of the Armadillo superfamily including β-catenin, which links them to the actin cytoskeleton via the α-catenin bridge (6, 7). In addition to its adhesive functions, β-catenin has also been found to serve as a key component in Wnt signaling (8, 9). When released from E-cadherin, uncomplexed β-catenin is rapidly degraded by cytosolic proteasomes. Failure to properly degrade β-catenin, primarily attributable to an impairment in its ubiquitination, results in its posttranslational stabilization and passage into the nucleus, where it interacts with transcription factors of T cell factor/lymphoid enhancer factor (Tcf/Lef) family to activate target genes involved in cell growth control and apoptosis such as c-myc and cyclin D1 (10, 11). Aberrant activation of β-catenin signaling has been implicated in cancer formation in numerous basic and clinical studies (12).

There is increasing evidence to suggest that phosphorylation of tyrosyl residues in some components of the cadherin/catenin complex leads to loss of adhesive function and breakdown of adherens junction. Roura et al. (13) reported that phosphorylation of tyrosine residue 654 on β-catenin diminishes its association with E-cadherin. Furthermore, they observed that phosphorylation of Tyr-654 also stimulated the association of β-catenin to the basal transcription factor TATA-binding protein. Thus, it is reasonable to conclude that phosphorylation-dependent release of β-catenin from the cadherin complex not only regulates the integrity and function of the adhesion complex but may also be an alternative mechanism for activating β-catenin signaling (14). We and others have previously shown that several RPTPs were functionally associated with E-cadherin/β-catenin complex and play a regulatory role in the control of the integrity of cell junctions, presumably by keeping them in a dephosphorylated state (15–18). In this study, we investigated the regulatory effect of PCP-2 on nuclear signaling activity of β-catenin. We demonstrate that PCP-2 repressed not only wild-type but also active mutant β-catenin-mediated transcriptional activity. PCP-2 expression in human colon carcinoma cell line SW-480, in which the β-catenin signaling pathway is up-regulated, led to stabilization of...
cytosolic pools of β-catenin, perhaps by their physical interaction and by enhancing adherens junction stability, thus decreasing the transcriptional activity of β-catenin by sequestering the protein at the plasma membrane. This effect was attributed to the inhibition of cell proliferation and migration when PCP-2 was transfected into SW480 cells. These results delineate a novel role for PCP-2 in regulation of the canonical β-catenin signaling pathway.

MATERIALS AND METHODS

Cell Culture and Transfections—HEK293, SW480 colon carcinoma cells, A431 human epidermoid carcinoma cells, and SW850 pancreatic carcinoma cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Stable SW480 clones were generated by transfection using JetPEI (Polyplus) of pCDNA3.0 or PCP-2 constructs and screened by Western blotting. Positive clones expressing similar levels of PCP-2 were cultured in the presence of 300 μg/ml G418 for maintenance of transgene expression.

Plasmids—Different recombinants for wild-type or mutant forms of PCP-2 were constructed using pCDNA3/Myc(-) (Invitrogen). All β-catenin constructs tagged with Myc were produced by PCR amplification with the use of human wild-type β-catenin in a pRK5RS vector as a template. The fidelity of the constructs was verified by DNA sequencing. The resulting PCR products were subcloned into the pCDNA3/Myc(-) vector. N-terminal-deleted β-catenin (β-ΔN) lacks the first 140 amino acids, C-terminal-deleted β-catenin (β-ΔC) lacks the last 147 amino acids, and armadillo-domain-deleted β-catenin (β-ΔArm) contains both the N-terminal 140 amino acids and the C-terminal 147 amino acids but lacks the 141–633 amino acids. Point mutant Y654E was obtained using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Antibodies—Monoclonal antibodies specific for E-cadherin and β-catenin were purchased from BD Transduction Laboratories and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Polyclonal antibodies against Myc tag (9B11) and phosphotyrosine (P-Tyr) were purchased from Cell Signaling Technology. The monoclonal antibodies specific for c-Myc was purchased from Neomarker (Fremont, CA).

β-Catenin/Tcf Luciferase Reporter Assay—Two different Tcf-luciferase reporter constructs were used in this study: an intact wild-type Tcf-luciferase construct (pGL-OT) and a mutant Tcf-luciferase reporter construct (pGL-OF) (both gifts of B. Vogelstein) (10). The cyclin D1 reporter plasmid was kindly provided by Dr. O. Tetsu (11). A Dual Luciferase reporter assay was carried out according to the manufacturer’s suggestions (Promega). pRL-TK (Promega) was cotransfected with each reporter construct to normalize for transfection. All experiments were performed in triplicate.

Immunofluorescence—Cells were cultured on glass coverslips, fixed with 3% paraformaldehyde in phosphate-buffered saline, and permeabilized with 0.2% Triton X-100. The cover slips were incubated with E-cadherin or β-catenin monoclonal antibodies at 4 °C overnight. The secondary antibody was fluorescein isothiocyanate- or Cy3-conjugated goat anti-mouse immunoglobulin G (Sigma). After being washed in Tris-buffered saline, coverslips were mounted with 90% glycerol in Tris-buffered saline and analyzed with a conventional fluorescence microscope (Olympus IX70).

Immunoprecipitation, Immunoblotting, and Chromatin Immunoprecipitation (ChIP)—Cells extracts were prepared using Nonidet P-40 lysis buffer (20 mM Tris–HCl, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 1 mM Na3VO4, 1 mM NaF, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin). The supernatant was collected and incubated with antibodies at 4 °C for 3 h and then with protein A for an additional 3 h. The beads were washed three times with lysis buffer and resuspended in SDS sample buffer. For immunoblot analysis, samples were separated by SDS-PAGE and transferred to nitrocellulose membrane (Schleicher & Schuell). The membrane was first probed with a specific antibody and then detected using the ECL system with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences). For ChIP assay, chromatin was isolated from formaldehyde-treated SW480 cells, fragmented to a mean size <600 bp, and subjected to ChIP using chromatin immunoprecipitation assay kits (Upstate Biotechnology, Hamburg, Germany) together with 10 μg of β-catenin-specific antibody following Upstate Biotechnology’s protocol. A specific primer pair for the c-myc promoter region was used for investigating the binding of β-catenin to DNA. For analyzing chromatin input, one-fiftieth of the precipitated chromatin was taken as a template, and for all other reactions, one-tenth of the precipitated chromatin was taken as a template.

Cell Fractionation—To obtain Triton X-100-soluble and -insoluble fractions, cells were incubated with Triton buffer (1% Triton X-100, 0.3 μM sucrose, 25 mM HEPES, pH 7.4, 100 mM NaCl, 4.7 mM KCl, 1.2 mM K2HPO4, 1.2 mM MgCl2, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) for 15 min on a rocking platform. After centrifugation, the supernatant (Triton X-100-soluble fraction) was collected. The cell pellet was resuspended in SDS lysis buffer (20 mM Tris, pH 7.5, 2.5 mM EDTA, 1% SDS, and 1 mM dithiothreitol) and subjected to standard SDS-PAGE and immunoblot analysis.

Aggregation Assay and Wound Healing—Cell-cell adhesion was evaluated in an aggregation assay, as described previously (19). In brief, cultures were rinsed with 10 mM HCMF buffer (10 mM HEPES, pH 7.4, 137 mM NaCl, 5.4 mM KCl, 0.3 mM Na2HPO4, 7H2O, 5.5 mM glucose) containing 2 mM CaCl2 and trypsinized into single cells by incubation with 0.04% trypsin in HCMF buffer supplemented with 2 mM CaCl2. They were then incubated under gyration shaking at 80 rpm for 30 min in HCMF containing 1% bovine serum albumin and 1.25 mM Ca2+ and photographed. For in vitro wound assays, monolayers of cells were wounded by scraping with a plastic pipette tip, rinsed several times with medium to remove dislodged cells, and cultured in serum-free Dulbecco’s modified Eagle’s medium for 24 h in a humidified incubator containing 5% CO2. Cells that had migrated into the wound area were photographed with a light microscope equipped with phase-contrast optics (Olympus IX70).

Cell Surface Biotinylation—Cells were rinsed once in serum-free Dulbecco’s modified Eagle’s medium and twice in ice-cold PBS, pH 7.5, and then incubated with 1.0 mg/ml sulfo-NHS-SS-biotin (biotin disulfide N-hydroxysuccinimide ester, Pierce) and dissolved in PBS for 30 min on a rocking platform on ice. Biotinylation was stopped by washing twice in PBS containing 100 mM glycine and twice in PBS for altogether 35 min. Cells were solubilized in lysis buffer (10 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, and protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin), pH 7.4. The unlysed cells were removed by centrifugation at 15,700 g for 30 min. The cell pellet was resuspended in SDS lysis buffer (20 mM Tris, pH 7.5, 2.5 mM EDTA, 1% SDS, and 1 mM dithiothreitol) and subjected to standard SDS-PAGE and immunoblot analysis.

Generation of Recombinant Adenovirus Expressing PCP-2—The PCP-2 coding sequence insert was cloned into the adenoviral shuttle vector (Stratagene). Then it was linearized with Pmel and cotransformed with E1-deleted adenoviral backbone AdEasy-1 into the competent bacterial
strain BJ5183, which allows efficient recombination to occur. After screening, recombinants for adenoviruses Ad-PCP-2 and Ad-Blank, which contain no insert sequence as a control, were generated.

Growth Curves and Colony Formation Assay—Growth Curves, stable SW480 cell lines (5 × 10⁴) were plated per well of 6-well plates. At each time point, the cells were trypsinized and counted. Each data point was performed in triplicate. The measurement of viable cell mass was also performed with a Cell Counting Kit-8 (Dojin Laboratories, Kumamoto, Japan) to count living cells by WST-8. For colony formation after transfection with drug selection, an equivalent number of SW480 cells (10⁶) were transfected with 2 μg of vector or PCP-2 constructs conferring neomycin drug resistance. After transfection, cells were replated and selected in G418-containing medium for 3 weeks, and the resultant colonies were fixed and stained with crystal violet. Transfections were done in triplicate for each combination of plasmids.

RNA Interference—Three double-stranded siRNA oligonucleotides against PCP-2 (sense strand, 5’-CCCAAAAGAAGAAGACAGGUCAA-3’, 5’-GGGACAUCAAGAUUAUGCUGGAA-3’, 5’-GAUGCGCAAUUGUCCAGAGUAUAUU-3’) were provided by Invitrogen. Lipofectamine 2000 (Invitrogen) was used as the transfection reagent according to the manufacturer’s directions with 150 nmol of siRNA per well in a six-well dish. A scrambled siRNA was used as the control. siRNA-transfected cells were incubated for 36–48 h in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum.
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FIGURE 2. PCP-2 reduced transactivation capacity of non-ubiquitinatable mutant forms of β-catenin. A, schematic presentation of the Myc-tagged point mutant β-catenin S37A and the different truncation forms of β-catenin (left); lysates from transfected HEK293 cells were analyzed by immunoblotting with anti-PCP-2 or anti-Myc antibodies. WB, Western blot. B, HEK293 cells were cotransfected with wild-type PCP-2 and the wild-type and mutant forms of β-catenin, together with the reporter plasmids. Luciferase activity was determined 24 h after transfection. pRL-TK was used as a control for transfection efficiency.

In Vivo Tumorigenicity Assay—SW480 transfectants were released from tissue culture dishes and washed in serum-free medium. Tumor cells were diluted with PBS and injected into the mid-dorsum of BALB/c nude mice (4–6 weeks old) in a total of volume 0.1 ml (5 x 10⁶). Animals were inspected weekly for tumor development. Growing tumors were measured using vernier calipers, and tumor volume was calculated of an elliptical solid. Statistical analysis was performed by Student’s t-test (two-tailed). The criterion for statistical significance was taken as p < 0.05. All procedures regarding animals were conducted according to institutionally approved protocols.

RESULTS

PCP-2 Inhibited β-Catenin-mediated Transcriptional Activity—We have previously demonstrated functional interaction between β-catenin and PCP-2. To study its effect on β-catenin-mediated transcription, PCP-2 was cotransfected with wild-type β-catenin into HEK293 cells together with Tcf reporter plasmid pGL-OT. As shown in Fig. 1A, PCP-2 very strongly suppressed β-catenin-mediated luciferase activity driven from the pGL-OT reporter in a dose-dependent manner. We also used LiCl, an inhibitor of GSK3β activity and widely used to mimic Wnt signaling, to increase endogenous β-catenin levels and detect its transactivation potential after transfection with PCP-2. Treatment of the cells with LiCl resulted in an evident increase in β-catenin levels. Although β-catenin activity was elevated in empty vector-transfected cells, this effect was significantly reduced in cells transfected with PCP-2 (Fig. 1B).

As functional β-catenin/Tcf-binding sites have been identified in the promoter of cyclin D1, we then tested the effect of PCP-2 on the cyclin D1 reporter transcription. Fig. 1C shows that cyclin D1 reporter was strongly transcribed in response to wild-type β-catenin. However, exogenous expression of PCP-2 imposed a substantial inhibition of the cyclin D1 promoter activity. To address the inhibitory effect of PCP-2 on β-catenin transcriptional activity under normal signaling conditions, we knocked down endogenous PCP-2 level using RNA interference strategy and then examined changes of β-catenin reporter gene activity after EGF stimulation in A431 human epidermoid carcinoma cells, which overexpress EGFR. As shown in Fig. 1, D and E, PCP-2 down-regulation by siRNA led to an increased transcriptional activity of β-catenin upon EGF treatment as compared with cells transfected with control siRNA. The specificity of PCP-2-mediated effects on Tcf reporters was confirmed by using pGL-OF, which harbors mutated Tcf-binding sites and was not influenced by PCP-2. Taken together, these results suggest that PCP-2 can exert specifically inhibitory effect on β-catenin-dependent transcriptional activity.

PCP-2 Down-regulated the Active Mutant Form of β-Catenin-induced Signaling—Mutations in the ubiquitin-targeting sequence of β-catenin occur in a number of different cancers (20). Among these mutations, S37A and N-terminal truncation are common stabilizing ones that render β-catenin resistant to ubiquitination-dependent degradation. To test whether PCP-2 could modulate the transcriptional activities of the degradation-resistant mutants of β-catenin, we cotransfected Myc-tagged wild-type and S37A, ΔN, ΔC, or ΔArm mutant β-catenin constructs (Fig. 2A) together with empty vector or PCP-2 and the Tcf reporter plasmids pGL-OT into HEK293 cells and monitored β-catenin signaling by assaying the reporter activity. As shown in Fig. 2B, transfection of S37A and ΔN mutant forms of β-catenin significantly increased the Tcf reporter activity in HEK293 cells, whereas ΔC and ΔArm did not, probably due to the loss of Tcf-binding sites and transcriptional domain of β-catenin. Cotransfection of PCP-2 significantly reduced both S37A and ΔN mutant forms of β-catenin-induced Tcf reporter activation, indicating that the inhibitory effect of PCP-2 on β-catenin is independent of its degradation sensitivity.
The Phosphatase Activity Was Involved in PCP-2-mediated Repression of \( \beta \)-Catenin Signaling—We previously showed that PCP-2 bound \( \beta \)-catenin and conferred its dephosphorylation (18). To examine whether the tyrosine phosphorylation of \( \beta \)-catenin was attributed to its transcriptional activity, we transiently transfected wild-type \( \beta \)-catenin or Y654E and Y654F mutant plasmids together with Tcf reporter constructs. Twenty-four hours later, Renilla-corrected \( \beta \)-catenin-dependent luciferase activity was determined. B, HEK293 cells were cotransfected with wild-type \( \beta \)-catenin or Y654E mutant and reporter constructs together with or without PCP-2. Shown is the fold repression of luciferase activity in the presence of PCP-2. Similar results were obtained in two additional independent experiments. C, HEK293 cells were cotransfected with reporter constructs, WT- or mu-PCP-2, and \( \beta \)-catenin. Renilla-corrected luciferase activity was measured 24 h after transfection. D, constructs for Myc-tagged wild-type PCP-2 or its phosphatase mutant form were cotransfected into HEK293 cells with wild-type \( \beta \)-catenin or Y654E mutant. Either wild-type or mutant PCP-2 proteins could be similarly detected in either wild-type or mutant \( \beta \)-catenin immunoprecipitates (IP). WB, Western blot.
β-catenin-Tcf-mediated transcription, and PCP-2-mediated dephosphorylation was necessary for its role in negatively regulating β-catenin signaling. To further confirm these results, a catalytically inactive mutant of PCP-2 (PCP-2/CS) was generated in which critical cysteine residues in both PTP domains were mutated to serine. As shown in Fig. 3C, introduction of the Cys to Ser mutation in both PTP domains evidently interfered with its inhibitory effect on β-catenin activity. To examine whether such mutations affect PCP-2 association with β-catenin, Myc-tagged wild-type or mutant PCP-2 were transiently cotransfected into HEK293 cells with wild-type or mutant PCP-2 (WT-PCP-2 or PCP-2/CS (mu-PCP-2) and characterized them by immunoblotting (Fig. 4A) and by immunofluorescence (Fig. 4B). SW480 cells were chosen for study because they harbor a truncated APC that renders β-catenin resistant to degradation and contains relatively low E-cadherin levels; thus, most of the β-catenin is not retained in the membrane by this molecule (21). Two independent clones for each construct with similar protein expression levels were used for further analysis. Drug-resistant clones of empty vector-transfected cells were pooled to rule out clone-specific effects. As shown in Fig. 4C, exogenous expression of WT-PCP-2 led to a substantial drop in Tcf reporter activities. The inhibitory effect of PCP-2 was partially compromised by inactivation of its catalytic activity, as expected from the above observation that the phosphatase activity was involved in PCP-2-mediated repression of β-catenin signaling (Fig. 3). To directly address whether there was physiological relevance to our finding of decreased β-catenin transactivation, we then evaluated the amounts of c-myc, which was a crucial target for β-catenin/Tcf4-mediated Wnt signaling activities. In Fig. 4, D and E, we showed that exogenous expression of PCP-2 or PCP-2/CS caused a significant reduction in the amounts of c-myc mRNA and protein levels, suggesting that the decreased transcriptional activity is accompanied by
specific decreases in gene transcription and that the decrease in c-Myc protein levels occurs at the mRNA level. A ChIP assay was further performed to determine whether PCP-2 signaling caused a direct effect on reducing binding of the β-catenin-Tcf transcription factor to the endogenous c-myc promoter region. Fig. 4F revealed that β-catenin was indeed recruited less to the promoter regions of c-myc gene in the PCP-2-expressing cell pool than in the empty vector control cell pool. These data demonstrate that PCP-2 induced dissociation of β-catenin-Tcf with c-myc promoter and suggest a new function for this RPTP in β-catenin-dependent gene regulation.

PCP-2 Directly Associated with β-Catenin and Enhanced Its Stability—To explore the molecular mechanism by which PCP-2 down-regulate β-catenin signaling activity, we first tested whether PCP-2 could associate with E-cadherin/β-catenin complex in SW480 cells. PCP-2 or β-catenin was immunoprecipitated from SW480 cells stably transfected with Myc-tagged WT- or mu-PCP-2. The immunoprecipitates (IP) were then analyzed by immunoblotting with anti-β-catenin, anti-E-cadherin, or anti-Myc tag antibodies. PC3, empty pcDNA3. WB, Western blot. C, cells were chased with cycloheximide (CHO) (50 μg/ml) to inhibit de novo protein synthesis. At the indicated times after the addition of cycloheximide, the levels of the β-catenin, E-cadherin, and β-actin proteins were analyzed at the indicated time points by cell lysis followed by Western blot analysis using the appropriate antibodies.

A
IP: myc (9B11)
PC3 WT1 CS9
-PCP-2-myc

B
IP: β-catenin
PC3 WT1 CS9
-β-catenin

C
CHX
0 3 6 9 h

WB: myc (9B11)

WB: β-catenin

WB: E-cadherin

WB: β-actin

FIGURE 5. Effect of PCP-2 on β-catenin stability. A and B, PCP-2 or β-catenin was immunoprecipitated from SW480 cells stably transfected with Myc-tagged WT- or mu-PCP-2. The immunoprecipitates (IP) were then analyzed by immunoblotting with anti-β-catenin, anti-E-cadherin, or anti-Myc tag antibodies. PC3, empty pcDNA3. WB, Western blot. C, cells were chased with cycloheximide (CHO) (50 μg/ml) to inhibit de novo protein synthesis. At the indicated times after the addition of cycloheximide, the levels of the β-catenin, E-cadherin, and β-actin proteins were analyzed at the indicated time points by cell lysis followed by Western blot analysis using the appropriate antibodies.

with cycloheximide to block new protein synthesis; protein extracts were prepared at 0, 3, 6, and 9 h after the block. Western blotting (Fig. 5C) showed that β-catenin was rapidly degraded in empty vector-transfected cells, whereas its levels remained stable in WT- and mu-PCP-2 cells. These data suggest that PCP-2 expression could lead to stabilization of cytosolic pools of β-catenin perhaps by virtue of their physical interaction.

Expression of PCP-2 Led to Decreased Free Cytoplasmic β-Catenin and Increased Membrane-associated E-cadherin—To determine whether the reduction in β-catenin/Tcf signaling was due to β-catenin sequestration by PCP-2 from the free competent signaling pool, β-catenin from SW480-derived cells was affinity-precipitated with a GST-E-cadherin cytoplasmic fusion protein, and the levels of free, uncomplexed β-catenin were analyzed by immunoblotting (Fig. 6A). As demonstrated previously, this strategy allows, in contrast to immunoprecipitation, specifically and selectively, the precipitation of the free, non-E-cadherin-bound pool of β-catenin (22). As shown in Fig. 6, the free, uncomplexed pool of β-catenin was significantly reduced in WT-PCP-2 cells as compared with control and mu-PCP-2 cells, whereas the total expression levels of β-catenin were unaffected. The data demonstrate that only WT-PCP-2 could stabilize or increase the E-cadherin-bound pool of β-catenin. To further characterize this effect, we evaluated the membrane-bound E-cadherin and β-catenin levels with a biotinylation method using sulfo-NHS-SS-biotin. Because this modified biotin is membrane-impermeable, it only binds to surface-associated proteins, which can then be separated from the intracellular pool by conjugation with streptavidin beads and visualized by Western blotting. Using this method, we observed increased surface expression of E-cadherin and membrane-bound β-catenin in WT-PCP-2 cells (Fig. 6B). These data, in conjunction with the results from the glutathione S-transferase pull-down studies, support the hypothesis that the functional association of PCP-2 with β-catenin is correlated with enhanced membrane localization of E-cadherin/β-catenin complex. It is noteworthy that membrane-bound β-catenin was also slightly increased in mu-PCP-2 cells. Because PCP-2 is a transmembrane protein, the increased recruitment of β-catenin at the cell-cell contacts in mu-PCP-2 cells was most possibly due to PCP-2 binding. Cell fractionation by Triton X-100 was further used to examine the attachment of the E-cadherin/catenins complex to the cytoskeleton. In Fig. 6C, we demonstrate that significantly increased amounts of E-cadherin and α- and β-catenin were localized in the Triton X-100-insoluble fraction in WT-PCP-2-expressing cells as compared with control cells, indicating that PCP-2 enforced the linkage of E-cadherin/catenins complex to cytoskeleton.

Consistently, forced expression of WT-PCP-2 but not mu-PCP-2 induced a striking rescue of normal epithelial morphology. The loose organization of the parent cells gave way to compacted epithelial colonies (Fig. 6D, panels a–c) with increased staining of E-cadherin and β-catenin at cell-cell junctions as observed by immunofluorescence analysis (Fig. 6D, panels d–i). Furthermore, aggregation assays show that WT-PCP-2-expressing cells formed large, tight cell clusters, whereas the other cell clones formed small aggregates that were poorly associated (Fig. 6E, panels a–c). Removal of calcium ions by the addition of EDTA/EGTA to WT-PCP-2-transfected cells decreased cell aggregation, which is comparable with the control or mu-PCP-2 cells (data not shown). These results demonstrate clearly that the increased membrane-associated E-cadherin in WT-PCP-2 cells was responsible for the observed cellular aggregation. The changes in cell adhesion led us to investigate the effect of PCP-2 expression on cell migration with use of a classical wound-healing assay. As expected, expression of WT-PCP-2 markedly reduced the number of migratory cells apparent 24 h after...
**FIGURE 6.** PCP-2 expression resulted in decreased free pool of β-catenin and increased adherens junction stability. 

A, affinity precipitations were performed with 5 μg of a GST-E-cadherin cytoplasmic protein. Precipitates and total cell lysate were separated by SDS-PAGE, and the levels of free, uncomplexed β-catenin as well as total levels of β-catenin were analyzed by Western blotting with the indicated antibodies. B, for cell surface expression (left panel), cells were biotinylated, and the labeled cell surface proteins were

|         | Triton-soluble | Triton-insoluble |
|---------|----------------|------------------|
| PC3     |                |                  |
| WT1     |                |                  |
| CS9     |                |                  |

|         | E-cadherin     | β-catenin        |
|---------|----------------|------------------|
| PC3     |                |                  |
| WT1     |                |                  |
| CS9     |                |                  |

**C**

|   | Free γ-catenin | Non-specific | Total γ-catenin |
|---|----------------|--------------|-----------------|
| PC3 |                |              |                 |
| WT1 |                |              |                 |
| CS9 |                |              |                 |

**D**

**E**

**Aggregation**

|   | PC3 | WT1 | CS9 |
|---|-----|-----|-----|
| a |     |     |     |
| b |     |     |     |
| c |     |     |     |

**Wound healing**

|   | PC3 | WT1 | CS9 |
|---|-----|-----|-----|
| d |     |     |     |
| e |     |     |     |
| f |     |     |     |

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initiation of the assay (Fig. 6E, panels d–g), suggesting that the tighter cell-cell adhesion of WT-PCP-2-expressing cells inhibits SW480 cell migration.

**PCP-2-mediated Dephosphorylation of β-Catenin Contributed to Enhanced Cell Adhesion**—Because the presence of phosphorylated tyrosine residues on β-catenin is correlated with the loss of E-cadherin-mediated cell adhesion, we then determined whether the level of phosphorylated β-catenin could be influenced by PCP-2 expression. As shown in Fig. 7A, the tyrosine phosphorylation of β-catenin was markedly attenuated in WT-PCP-2 cells without any overall change in the β-catenin steady-state level after exposure to EGF, suggesting that expression of WT-PCP-2 shifts the balance of the tyrosine phosphorylation/dephosphorylation of β-catenin in favor of the latter. The amount of E-cadherin coimmunoprecipitated with β-catenin dramatically increased as the proportion of tyrosine-phosphorylated β-catenin declined, suggesting that abrogation of β-catenin tyrosine phosphorylation markedly enhanced β-catenin-E-cadherin association (Fig. 7A).

Although β-catenin itself appeared as the major site of PCP-2 action, it was possible that the restriction to β-catenin phosphorylation might be secondary to impaired β-catenin-associated activation of receptor protein-tyrosine kinase. This possibility was examined by analysis of EGFR phosphorylation and subsequent Erk activation upon EGF treatment. As shown in Fig. 7B, the response of EGFR to EGF stimulation was independent of PCP-2 expression, and Erk activation also behaved similarly. Therefore, EGFR regulation might not be the key mechanism underlying the effects of PCP-2 on β-catenin. Taken together, our data suggest that PCP-2 represents a specific negative regulator of β-catenin tyrosine phosphorylation, which induces E-cadherin/β-catenin association and prevents an increase in free β-catenin, thereby inhibiting its signaling activity.

**PCP-2 Inhibited Cell Growth and Transformation in SW480 Cells**—On the basis of the above observation, we then determined whether downregulation of the β-catenin signaling pathway by PCP-2 affected cell growth and transformation in SW480 cells. As compared with empty vector or the PCP-2 mutant, introduction of WT-PCP-2 significantly inhibited cell growth and colony formation (Fig. 8, A and C). Interestingly, mu-PCP-2 cells showed an intermediate suppression between control and WT-PCP-2 cells. We also constructed Ad-Blank and Ad-PCP-2 adenovirus vectors under the control of the cytomegalovirus promoter. Cells were infected with the adenoviruses, and the cell growth rate was measured using CCK-8 cell proliferation assay. Ninety percent of cells were infected at multiplicity of infection of 30, as judged by immunofluorescence analysis of PCP-2 expression (not shown). As shown in Fig. 8B, the growth rate of SW850 pancreatic carcinoma cells, in which β-catenin/Tcf-mediated transcription was inactivated, was not affected by Ad-PCP-2, whereas SW480 growth was significantly retarded (Fig. 8A and not shown). These data suggest that the effects of PCP-2 on growth were mediated, even in part, through regulation of β-catenin activity. Suppression of cell transformation following the exogenous PCP-2 expression in SW480 cells was confirmed in tumor xenografts (Fig. 8D). The tumor growth rate for vector control cells injected into nude mice is shown, whereas WT-PCP-2 cells did not grow tumors. Thus, stable expression of WT-PCP-2 in SW480 cells is sufficient to eradicate the tumorigenic growth of SW480 colon cancer cells, implicating a role for PCP-2 in counteracting β-catenin-mediated neoplastic transformation even in the presence of oncogenic mutations in APC.

**DISCUSSION**

In this study, we have identified PCP-2 as a novel PTP of adherens junction. Our results are along the lines of the general view that activation of PTPs stabilizes the cadherin-catenin complex and results in increased cadherin-mediated cell-cell adhesion but go further in showing that PCP-2 also has the potential to suppress β-catenin-dependent signaling activity. There are at least two different mechanisms for negatively regulating β-catenin signaling: the APC/GSK3β/axin-dependent destruction pathway and a cadherin-dependent sequestration pathway (21, 23, 24). Our observation that PCP-2-induced inhibition of...
β-catenin signaling was irrespective of its proteasome sensitivity indicates that the forenamed destruction pathway might, seemingly, not be responsible for this effect. In general, activation of tyrosine kinases results in a loss of cadherin-mediated cell-cell adhesion and an increase in the level of cytoplasmic β-catenin. For example, activation of EGFR or c-Met receptor tyrosine kinase resulted in β-catenin tyrosine phosphorylation, accumulation, and increased β-catenin-mediated gene transcription (25, 26). Conversely, pharmacological depletion of ErbB2 resulted in increased binding of β-catenin to cadherin and a consequent decrease in β-catenin-mediated gene transcription (27). Because PCP-2 could directly associate with β-catenin and confer its dephosphorylation, we therefore speculate that PCP-2 might repress β-catenin signaling either by directly sequestering cytosolic β-catenin or by indirectly stabilizing E-cadherin/β-catenin complex to plasma membrane. We indeed found that loss of catalytic activity of PCP-2 reduced its inhibitory effect on β-catenin-mediated transactivation, suggesting that the inhibitory effect on β-catenin signaling activity was not only due to direct association with PCP-2. After induction of wild-type PCP-2 expression in SW480 cells, membrane-associated E-cadherin/β-catenin complex was increased, and the free, uncomplexed β-catenin was decreased. Furthermore, the levels of phosphotyrosine content of β-catenin were evidently decreased in the presence of wild-type PCP-2 upon EGF stimulation, confirming that β-catenin may act as a substrate of PCP-2. In contrast, using a construct that can bind β-catenin but was defective in phosphatase activity had little effect on E-cadherin localization. Although the association of mutant PCP-2 and β-catenin also led to slightly increased levels of membrane-associated β-catenin and reduced β-catenin turnover, the inhibition of β-catenin signaling activity was significantly attenuated in mu-PCP-2-expressing cells. These results suggest that direct binding of β-catenin by PCP-2 may not only sequester free cytoplasmic β-catenin in the vicinity of cell membrane but also induce its subsequent dephosphorylation and association with E-cadherin, thereby increasing the stability of adherens junctions, thus making β-catenin unavailable for signaling to the nucleus.

We also found that enforced expression of PCP-2 in colon carcinoma cell line SW480 significantly suppressed cell proliferation and migration. Growth inhibition of PCP-2 correlated with a reduction in β-catenin signaling, as assessed with β-catenin/Tcf-responsive reporter assay.

**FIGURE 8.** PCP-2 inhibited cell growth and transformation in SW480 cells. A, growth curves for stable SW480 transfectants in complete medium containing 10% serum. Each measurement was the mean of cell counts from at least three wells. B, SW850 cells in 96-well plates were infected with Ad-Blank or Ad-PCP-2 adenoviruses at a multiplicity of infection of 30. Cell proliferation was measured using CCK-8 assay at the indicated times. The number of viable cells is proportional to the color intensities. The data shown are the means of five wells for each condition. This experiment has been repeated once, and the result was consistent with data shown here. C, colony formation assay after transfection with control plasmid (pcDNA3), wild-type PCP-2, or its mutant construct. Mock, mock-transfected. D, Xenograft tumor growth rate. 5 × 10⁶ cells were injected subcutaneously into nude BALB/c mice (n = 5/group), and tumor volumes of xenografts were measured. Shown is the mean (±S.D.) tumor volume for SW480-pcDNA3 cells and SW480-WT-PCP-2 cells.
and evidenced by consequent inhibition of β-catenin target gene, c-myc. Association of tyrosine-dephosphorylated β-catenin with E-cadherin was enhanced, and this occurred concurrently with dramatically reduced cell motility, which seems to be primarily a consequence of changes in cell adhesion. Because PCP-2 was able to mediate cellular homophilic binding in a calcium-independent manner, it is tempting to assume a dual function for this molecule in contributing directly to the adhesion of cells by its extracellular domains and indirectly via regulation of the tyrosine phosphorylation balance at cell adhesion junctions by their intracellular domains. However, aggregation assays revealed that cells expressing WT-PCP-2 were unable to form the tightly compacted cellular aggregates following treatment with calcium depletion. We therefore postulate that PCP-2-induced E-cadherin membrane localization is mainly responsible for the enforced cell integrity, and PCP-2 appears to cooperate with E-cadherin to facilitate this process.

Although β-catenin itself appears as the major site of PCP-2 action, the mechanism whereby PCP-2 confers E-cadherin/β-catenin stabilization also could result from its modulation on activities of β-catenin-associated tyrosine kinases. For example, PTP-LAR (leukocyte common antigen-related) and CD45 have been shown capable of attenuating the mechanism whereby PCP-2 confers E-cadherin/Catenin Signaling by PCP-2.

Given the ability of PCP-2 to suppress both proliferation and migration, carcinomas that lose PCP-2 expression or function may acquire a more aggressive behavior. In support of this hypothesis, a mutational analysis of the tyrosine phosphatase gene superfamily in human colon cancers identified frequent somatic mutations in PTPrp, another member of the MAM-PTPs family. Importantly, most commonly altered PTPrp genes were found to reduce phosphatase activity, and expression of wild-type, but not a mutant, PTPrp in human cancer cells inhibited cell growth, which is strikingly consistent with our results. In addition, PCP-2 (also known as PTPrp) was recently shown to be down-regulated in melanoma, which has long been recognized as the highly metastatic tumor (31). It is of note that melanoma also contains highly tyrosine-phosphorylated β-catenin and, importantly, frequent stabilizing mutations in β-catenin or inactivation of APC (27, 32). Thus, it will be interesting to determine the therapeutic value of reintroduction of PCP-2 into these melanoma cells.

Recently, Aerne and Ish-Horowicz (33) reported that reduction of PCP-2 activity resulted in severe disruption of the segmental pattern of the zebrafish embryo and interfered with convergent extension during gastrulation. In light of our results, it is therefore possible that reduced PCP-2 activity may impinge both on the formation and establishment of specific cell layers during morphogenesis of convergent extension movements and on β-catenin-directed transcriptional regulation of the segmentation. However, the mechanistic insight into this possibility warrants further study.

In summary, the results presented in this study suggest that PCP-2-mediated sequestration of β-catenin and abrogation of its tyrosine phosphorylation, even in cells expressing aberrantly stable β-catenin, may counteract the tumor-promoting activity of nuclear-localized β-catenin while simultaneously enhancing the tumor suppressive activity of plasma membrane β-catenin-E-cadherin complexes.

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