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Biochemical and Biophysical Research Communications 349 (2006) 200–208
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Prenylated Rab acceptor 1 (PRA1) inhibits TCF/β-catenin signaling by binding to β-catenin

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Received 11 July 2006
Available online 14 August 2006

Abstract

The prenylated Rab acceptor 1 (PRA1) is a ubiquitously expressed 21 kDa protein containing two transmembrane domains that possibly induce its localization to the Golgi complex. It binds to prenylated Rab GTPases and VAMP2. In this study, we report that PRA1-overexpressing cells exhibited a significantly retarded growth rate as compared to that of the mock-transfected cells, and the transcriptional activity of TCF, as evaluated by TOPflash luciferase reporter assay, was profoundly reduced in the PRA1-overexpressed cells. These intracellular functions of PRA1 were verified by introducing deletion mutant or site-directed mutants, or small interfering RNA of PRA1. In addition, the translocation of β-catenin from the cytosol to the nucleus was blocked to a significant degree in the PRA1-cells, and the interaction of PRA1 and β-catenin was identified by confocal microscopy and immunoprecipitation analysis. Finally, we observed that the inhibition of TCF/β-catenin signaling by PRA1 is associated with ERK1/2 dephosphorylation. Therefore, our data suggest that the *in vivo* modulation of PRA1 may be involved in TCF/β-catenin signaling, as well as cellular proliferation and tumorigenesis.

Keywords: Prenylated Rab acceptor 1 (PRA1); Luciferase reporter assay; TCF; β-Catenin signaling

Prenylated Rab acceptor 1 (PRA1) containing two transmembrane domains is a 21-kDa protein that is ubiquitously expressed in most tissues, and localized in the Golgi complex. PRA1 was initially identified as a Rab-GTPase-interacting protein in a yeast two-hybrid screen, showing that it binds to the prenylated Rab GTPase proteins including Rab1, Rab3, and Rab5 [1,2]. These GTPases, which are Ras superfamily GTPases, are small proteins (<25 kDa) that bind to guanosine triphosphate (GTP) [3–5], and regulate a broad range of cellular processes, including cell growth, differentiation, and movement. They also regulate cellular vesicle trafficking, which includes vesicle budding, tethering, and fusion. Rab GTPases play important regulatory roles in motility, via the recruitment of motor proteins to various organelles, and also coordinate intracellular signaling events associated with membrane trafficking [6–8]. All regulatory GTPases employ a common mechanism that enables them to switch on a signal transduction cascade via the unidirectional alteration of the GTPase from the active GTP-bound to the inactive GDP-bound state by hydrolysis of the GTP [9–11]. It has been shown that PRA1 may be involved in the vesicle trafficking from the endoplasmic reticulum (ER) to the Golgi complex. Because PRA1 is primarily associated with GTPases exhibiting a variety of biological effects, the elucidation of intracellular PRA1

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doi:10.1016/j.bbrc.2006.08.026

functions is required in order to gain insight into the sophisticated molecular network of Rab GTPases.

The interaction of GTPases with PRA1 requires their post-translational modification by prenylation [1,2,12]. Prenylation is followed by the proteolysis of the C-terminal tripeptide and the methylation of the newly generated C-terminal amino acid within the ER compartment [13]. Prenylated-GTPases interact with PRA1, which functions as an escort protein for GTPases and facilitates their translocation to the target membranes. PRA1 has been shown to associate with the GDP dissociation inhibitor (GDI), which solubilizes GTPases in the membrane [14]. Therefore, PRA1 and GDI exert opposing effects on Rab GTPases in regard to membrane targeting. Moreover, PRA1 has been shown to bind directly to vesicle-associated membrane protein 2 (VAMP2), which is involved in both the targeting and fusion of transport vesicles to their target membranes [1,15]. The dissociation of PRA1-VAMP2 may facilitate vesicle fusion and transport to the membrane. In addition, PRA1 has been shown to play roles in anti-apoptotic processes and viral infectivity by interacting with various viral proteins, including the CaMV movement protein [16], the Epstein–Barr virus BHRF1 [17], the rotavirus V4 spike protein [18], and the SIV gp41 [19].

The intracellular localization of PRA1 and its associated molecules has been relatively well characterized, but the intracellular functions of PRA1 remained to be elucidated. Interestingly, when PRA1 was overexpressed in HEK293 and HCT116 cells, cell growth was arrested, and cell proliferation was substantially inhibited. The results from cells transfected with various mutants or small interfering RNA of PRA1 suggested that Golgi-localized PRA1 might be essential for the inhibition of Wnt signaling. Collectively, the data suggest that PRA1 is a potential regulator in T cell factor (TCF)/β-catenin signaling, and that its modulation may play a crucial role in the transmission of cellular signals related to tumorigenesis.

Materials and methods

Cell culture and treatment. The HEK293 and HCT116 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY) supplemented with 2 mM glutamine, 1% penicillin/streptomycin, and 10% fetal bovine serum (FBS; HyClone, Logan, UT) at 37 °C in a CO_2 incubator. LiCl (Sigma, St. Louis, MO) at 20 mM concentration was added to the cell culture for 6–12 h after transfection. The inhibitors, including LY294002 for PI3K, PD98059 for ERK1/2, and SB203580 for p38 kinase, were all purchased from Sigma, and administered at a concentration of 20 μM. The AKT-I used for the AKT kinase inhibition was purchased from Calbiochem (San Diego, CA).

Antibodies. Mouse anti-GFP mAb was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-β-catenin and anti-N-cadherin mAbs were obtained from Upstate Biotechnology (Lake Placid, NY). Anti-HA, anti-Flag, and anti-α-tubulin mAbs were purchased from Sigma. The rabbit anti-phospho-GSK3β, anti-phospho-ERK1/2, and anti-ERK1/2 Abs were all acquired from Cell Signaling Technology (Beverly, MA). The horseradish peroxidase-conjugated anti-mouse or anti-rabbit Ab and the TRITC-conjugated anti-mouse Ab were obtained from Sigma. Polyclonal anti-PRA1 Ab was generated by injecting the recombinant PRA1 protein into mouse.

Plasmids. The full-length cDNA for human PRA1 (185aa, NM_006423) was cloned from a Jurkat cDNA library via PCR amplification. The deletion or site-directed mutants of PRAΔTM (Δ65-164aa), PRAΔN (Δ1-61aa), PRAΔ^{185}V, PRA-V185E, and PRA-DGAA (DGEE substitution) were generated via PCR, and the constructed plasmids were verified by their sequences. β-catenin (781aa) and β-cateninΔ1–47 (a deletion mutant of N-terminal 1–47 amino acids) were also cloned into the pcDNA3 expression vector.

Transfection, immunoprecipitation, and Western blotting. Cells at 80% confluence (50% for siRNA) were plated one day before transfection, then transfected with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The amount of DNA or siRNA for transfection was used at 1.5 μg or 30 μM per well in a six-well plate, respectively. The Stealth siRNA specific for PRA1 was generated from Invitrogen (PRA1; sense 5′-GCGCCUGUUACAUUCUCUAUCUGCG-3′). After 1–4 days, the transfected cells were washed and harvested for the respective experiments. In the Western blotting, the cells were washed with phosphate-buffered saline (PBS) and lysed with a lysis buffer [20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM Na_3VO_4/NaF, and protease inhibitors cocktail (Sigma)] on ice for 30 min. The resultant lysates were then clarified by centrifugation and the total protein content in the cell lysates was quantified via Bradford assay. For immunoprecipitation assay, the lysates were incubated with anti-HA mAb and protein-A/G–agarose at 4 °C for 4 h, and the immune complexes were resuspended with SDS sample buffer. The lysates or precipitates were then loaded onto 10–14% SDS–PAGE gels, transferred to PVDF membranes, and the membranes were blocked with 5% non-fat dried skim milk in TBS (20 mM Tris–HCl, pH 7.4, 150 mM NaCl) at room temperature. After incubation with the appropriate primary Abs for 4 h, the membranes were incubated with HRP-conjugated secondary Abs and visualized with an ECL® detection system (Amersham Pharmacia Biotech, Piscataway, NJ).

Nuclear fractionation. Cells were transfected for 1.5 days, and then harvested after washing with cold PBS. In order to segregate the nuclear fractions from the cell lysates, cell pellets were resuspended in TM buffer (10 mM Tris–HCl, pH 7.4, 2 mM $MgCl_2$, and 0.4 mM PMSF) containing protease inhibitors cocktail, and then allowed to react in 0.5% Triton X-100 on ice for 20 min. After the lysates were centrifuged at 1500 rpm for 5 min, the pellets harboring the nuclear fractions were resuspended using SDS sample buffer, and the supernatants obtained from a further 5 min of centrifugation at 5000 rpm were employed in the detection of cytosolic proteins.

Cell proliferation and luciferase reporter assay. The MTS reduction assay (Promega, Madison, WI) was performed in a 96-well plate according to the manufacturer's instructions. Briefly, cells were transfected with 0.2 μg DNA per well, and after 1–4 days MTS/PMS solution was added to each of the 96-well plates. After incubation at 37 °C in a CO_2 incubator for 1 h, the absorbance at 490 nm was recorded with a plate reader. In order to conduct the luciferase reporter assay, the cells were transfected with TOPflash (or FOPflash) luciferase reporter plasmid (Upstate Biotechnology), β-galactosidase plasmid, and pcDNA3(HA)-construct plasmid at 1.5 μg/well. β-Galactosidase was used to normalize the transfection efficiency of the cells. After two days, the cells were lysed with lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 1% Triton X-100) on ice for 30 min, and the cleared lysates were then transferred to each of the wells in the 96-well plates, and luciferase assay reagent was added. The light intensity of the reaction was determined using a plate-reading luminometer (Turner Designs, Sunnyvale, CA) and the luciferase intensity was calculated relative to the β-galactosidase activity.

Confocal microscopy. The cells were cultured on coverslips one day prior to transfection and were transfected with plasmids for 1.5 days. They were then rinsed three times in cold PBS, fixed with 4% paraformaldehyde at room temperature for 20 min, washed, and permeabilized with 0.1% Triton X-100/PBS for 10 min. Next, the cells were blocked with 1% BSA/PBS for 30 min and stained with anti-β-catenin Ab for 2 h. Finally, the cells were incubated with TRITC-conjugated rabbit anti-mouse Ab in darkness for 1 h, and the nuclei were stained with DAPI. The coverslips containing the cells were mounted on glass slides with VectaShield

mounting medium (Vector Laboratories, Burlingame, CA) and visualized using a Zeiss confocal microscope LSM510META (Carl Zeiss, Jena, Germany) at 40× magnification. The confocal images were captured by the Zeiss LSM Image Browser program.

RT-PCR analysis. Four groups of tissue pairs, including normal and cancerous region from patients with colon cancer, were obtained from the tumor tissue bank of local hospital. Prepared colon cancer tissues were lysed, and total RNA was extracted by using TRIzol reagent (Invitrogen) according to manufacturer's instructions. The RNAs were quantified, and 5 μg RNA was used for RT-PCR to generate each cDNA sample using a ProSTAR First-strand RT-PCR kit (Stratagene, La Jolla, CA). β-Actin product was used as a reaction standard and human PRA1 product was obtained using a specific primer sequence (sense: 5′-ATGGCAG CGCAGAAGGACCAG-3′, antisense: 5′-TCACACGGGTTCCATCTG CAG-3′).

Results

PRA1-overexpressing cells exhibit growth retardation

While PRA1 has been identified as an acceptor of Rab GTPase proteins, and the cellular localization of PRA1 and its associated molecules has been relatively well characterized, the intracellular functions of PRA1 remain to be elucidated. To gain insight into the intracellular functions of PRA1, the PRA1 gene, harboring the full-length cDNA, was transfected into HEK293 cells. PRA1 protein contains two transmembrane (TM) domains and the di-acidic DGEE motif at the C-terminal region as shown in Fig. 1A. It has been shown that the TM domains determine PRA1's cellular localization to the Golgi complex and the di-acidic motif is important for the localization from ER to the Golgi complex [20]. Interestingly, the cell growth rate, as evaluated by MTS reduction assay, was reduced substantially in the PRA1-overexpressing cells (Fig. 1B). However, the cells transfected with PRAΔTM, a TM-deleted mutant, showed no significant change in the growth rate when compared to a mock control. Also, we compared the growth rate of the Golgi-localized PRA1 wild-type and ER-localized PRA1 mutants such as PRAΔ^{185}V (185Val deletion) and PRA-DGAA (substitution of DGEE), but the growth rate was similar, regardless of PRA1's localization (data not shown). Therefore, the data suggest that PRA1 works in the conformation of a membrane-bound state and may play a role in cellular proliferation.

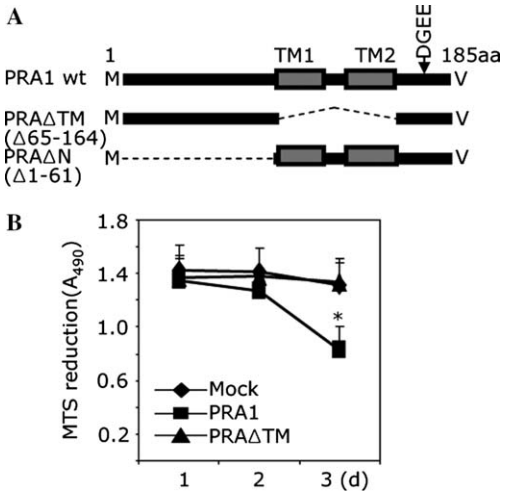

Fig. 1. Growth arrest in PRA1-overexpressing cells. (A) Schematic representation of PRA1 wt and its deletion mutants. PRA1 has two transmembrane (TM) domains and a di-acidic DGEE motif at the C-terminus. (B) HEK293 cells were transfected with pcDNA3HA or pcDNA3HA-PRA1 wt (or PRAΔTM). Cell proliferation was assessed via MTS assay 1–3 days after transfection. PRA1-overexpressing cells, but not PRAΔTM cells, exhibited growth arrest. Data are represented as means ± SE from three independent experiments. * $p < 0.005$ versus mock control.

PRA1 inhibits TCF/β-catenin signaling

In an attempt to gain insight into the mechanisms associated with cell growth inhibition by PRA1 overexpression, we examined whether PRA1 can modulate TCF/β-catenin signaling. The TCF/β-catenin complex is formed in cytosol by intracellular or extracellular signals and then translocates to the nucleus, functioning as a transcription factor for differentiation and proliferation. To analyze these modulations, we used the TOPflash luciferase reporter assay, which uses a luciferase reporter plasmid with three copies of the optimal TCF/lymphoid enhancer factor (LEF) binding site upstream of the minimal thymidine kinase (TK) promoter. The HEK293 cells were transfected with TOPflash (or FOPflash, which harbors mutant TCF binding sites) luciferase reporter, β-galactosidase (for the quantification of transfection efficiency), and PRA1 (PRA1 wt, PRAΔTM, PRAΔN, PRAΔ^{185}V, PRA-V185E, or PRA-DGAA) plasmids. The PRA1-transfected cell lysates exhibited very low levels of luciferase activity as compared with the vector controls, and this degree of intensity was similar to that evidenced by PRAΔ^{185}V, PRA-V185E, or PRA-DGAA (Fig. 2A). However, PRAΔTM and PRAΔN were not able to inhibit the TOPflash luciferase activity, indicating that full-length form including the TM and N-terminal regions is necessary for inhibiting the activity. FOPflash luciferase activity was not altered by PRA1 expression.

In both canonical and non-canonical Wnt/β-catenin signaling, the transport of the TCF/β-catenin complex to the nucleus is an essential step with regard to the transcriptional activity of genes associated with proliferation [21,22]. Therefore, we attempted to determine whether or not PRA1 inhibits TCF/β-catenin signaling under conditions in which β-catenin is exogenously expressed. As shown in Fig. 2B, β-catenin induced an elevated TCF/β-catenin signaling activity, but the TCF/β-catenin signaling activity was significantly inhibited in the cells co-transfected with PRA1. In contrast, the cells co-transfected with PRAΔTM and β-catenin did not show any reduction of the TCF activity. The activity of TCF signal by α-catenin introduction appeared to occur at lower levels than that in the vector control, indicating that an interaction between α-catenin and β-catenin results in the inhibition of basal β-catenin signaling. We also determined the effects of LiCl

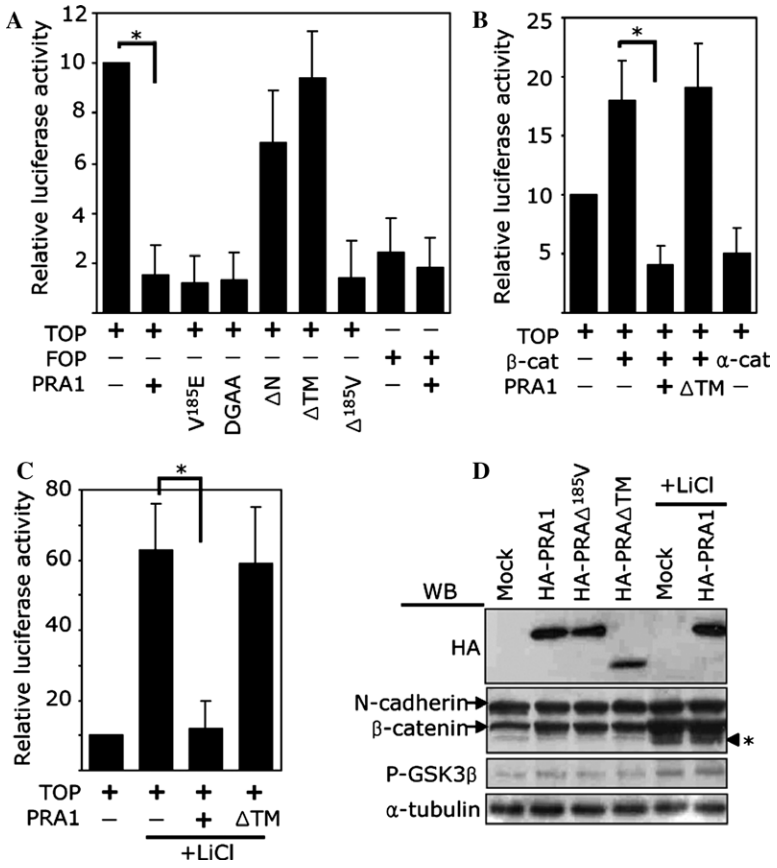

Fig. 2. PRA1 inhibits TCF/β-catenin signaling. (A) HEK293 cells transfected with TOPflash luciferase reporter (or FOPflash), pcDNA3HA-PRA1 variants, and β-galactosidase plasmid (to quantify the transfection efficiency) were cultured for 1–2 days. The cell lysates were assayed for the luciferase intensity using a luminometer. The intensity was calculated as values relative to β-galactosidase activity. TOPflash reporter activity was inhibited by PRA1 as well as PRA-V185E, PRA-DGAA, and PRAΔ^{185}V, but not PRAΔN and PRAΔTM cells. The activity of FOPflash reporter as a control plasmid was not affected by PRA1. * $p < 0.005$. (B) Cells were transfected with TOPflash, β-catenin (or α-catenin), or PRA1 wt (or PRAΔTM). TCF activation by β-catenin was inhibited in PRA1-cells, but not in PRAΔTM cells. * $p < 0.005$. (C) Cells were transfected with TOPflash and PRA1 wt (or PRAΔTM) for 1 day and treated with LiCl (20 mM) for 12 h. Data are represented as means ± SE from three independent experiments. * $p < 0.005$. (D) Cells were transfected and further treated with or without LiCl (20 mM) for 12 h. Cell lysates were subjected to SDS–PAGE for Western blotting to assess β-catenin expression and GSK3β phosphorylation. N-cadherin and α-tubulin were used as internal protein controls and β-catenin degradation is indicated by the asterisk. Results are from a single analysis, representative of two independent experiments.

on PRA1-related TCF/β-catenin signaling, because the treatment of LiCl is known to inhibit GSK3β and mimic Wnt signaling via the stabilization of β-catenin [23]. LiCl-activated TCF/β-catenin signaling approximately six-fold, whereas the exogenously introduced PRA1 completely inhibited LiCl-activated TCF/β-catenin signaling (Fig. 2C). However, TCF/β-catenin signaling by LiCl was not inhibited by PRAΔTM. Subsequently, we tested the stability and intracellular expression levels of β-catenin via Western blotting for β-catenin and glycogen synthase kinase (GSK) 3β. The stability of β-catenin and GSK3β was not affected by PRA1 overexpression, whereas LiCl stimulation resulted in a marked increase in the β-catenin concentration and a slight increase of GSK3β phosphorylation, but N-cadherin expression was not changed (Fig. 2D). The constant expression of phosphorylated-GSK3β and β-catenin indicates the possibility that PRA1 does not directly affect the stability of β-catenin. Furthermore, the data obtained using the deletion mutants suggest that the TM domain of PRA1, responsible for membrane localization to the ER or Golgi complex, is crucial for PRA1 activity with regard to TCF/β-catenin signaling.

PRA1 blocks the nuclear translocation of β-catenin

Because PRA1 was shown to inhibit the TCF reporter activity, we examined whether the translocation of β-catenin to the nucleus is blocked by PRA1. To do this, we generated the constructs for expression of an Enhanced Green Fluorescent Protein (EGFP)-fused wild-type and mutant PRA1. PRA1 was mainly localized to the Golgi complex, whereas PRAΔTM was distributed at a higher density in the nucleus and weakly in cytosol (Fig. 3A). To compare the β-catenin concentration in the cytosol and the nucleus, EGFP and EGFP-PRA1 variants were transfected and lysates were prepared. Cellular fractionation showed that the nuclear condensation of β-catenin in the EGFP-PRA1- and EGFP-PRAΔ^{185}V-cells was diminished

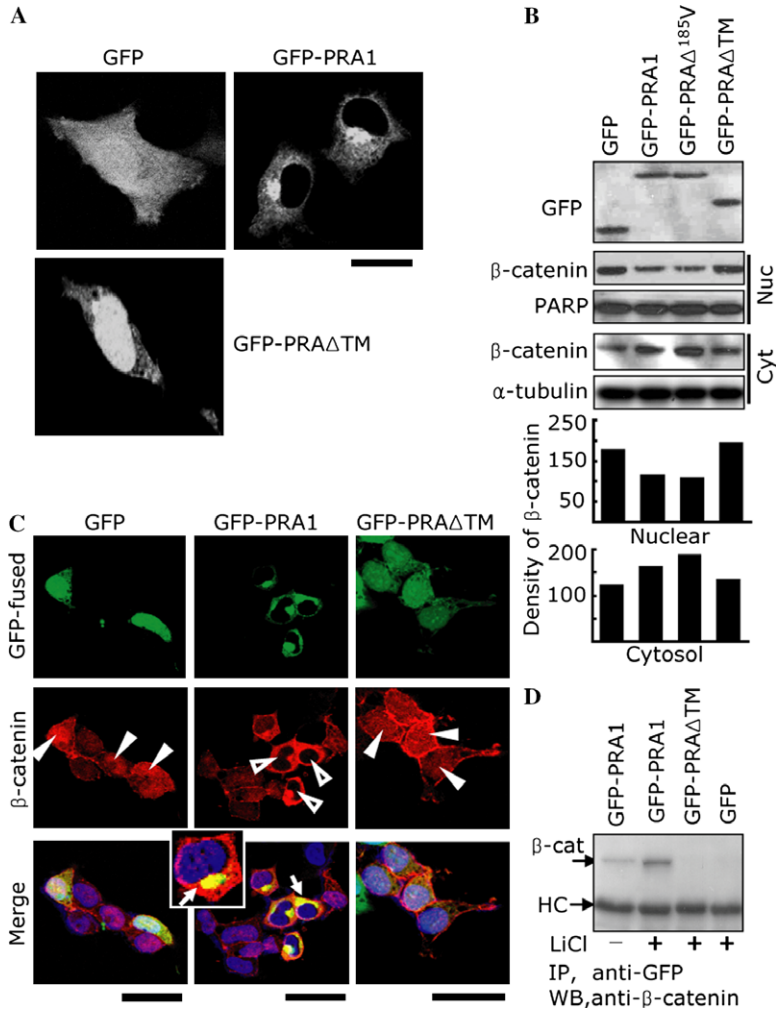

Fig. 3. The nuclear translocation of β-catenin is blocked by PRA1. (A) Cells on coverslips were transfected with EGFP or EGFP-PRA1 wt (or PRAΔTM), fixed, mounted on microscope slides, and EGFP-PRA1 expression was visualized. Scale bar, 25 μm. (B) Cells were transfected with EGFP-constructs and lysed for cellular fractionation as described in Materials and methods. PARP and α-tubulin were used as internal controls to detect the nuclear and cytosolic protein, respectively. Also, the density of β-catenin bands was scanned by a densitometer. (C) Cells on coverslips were transfected with EGFP, EGFP-PRA1 or EGFP-PRAΔTM, and treated with LiCl (20 mM) for 6 h. Cells were fixed and stained with anti-β-catenin Ab and DAPI for nuclear staining. Solid arrowheads indicate the nuclear condensation of β-catenin and open arrowheads indicate the nucleus where β-catenin is not translocated. As shown in merged images of EGFP-PRA1 column (especially in inset), EGFP-PRA1 and β-catenin were colocalized (indicated with arrows, yellow regions). Scale bar, 50 μm. (D) After cells were transfected with EGFP, EGFP-PRA1 or EGFP-PRAΔTM for 1 day, they were treated with LiCl as in (C). Cell lysates were immunoprecipitated with anti-GFP Ab and the immune complexes were analyzed by Western blotting against β-catenin. β-Catenin was precipitated in PRA1-containing lysates, but not in GFP alone or PRAΔTM. HC, heavy chain. (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)

(Fig. 3B). Consistent with this observation, β-catenin was strongly retained in the cytosol of both the PRA1 and PRAΔ^{185}V-cells, but PRAΔTM induced no detectable change in β-catenin level. Also, using confocal microscope, we investigated the β-catenin translocation to the nucleus in PRA1-transfected HEK293 cells. Cells transfected with the EGFP, EGFP-PRA1, or EGFP-PRAΔTM were treated with 20 mM LiCl for 6 h and then stained with anti-β-catenin Ab and DAPI. Immunofluorescence staining revealed the distribution of β-catenin in the nuclei of the EGFP- or EGFP-PRAΔTM cells, as indicated by the solid arrowheads (Fig. 3C). In contrast, most of the β-catenin in the EGFP-PRA1-expressing cells clearly accumulated in the cytosol, and the nuclear translocation of β-catenin was severely hampered as indicated by the open arrowheads. Interestingly, we also found that PRA1 and β-catenin were colocalized near the nucleus, likely at the Golgi complex (indicated by arrows, yellow). This suggests the possibility that PRA1 may interact with β-catenin. To verify its interaction, we performed immunoprecipitation assay from the lysates of cells transfected with EGFP or EGFP-PRA1 with or without LiCl. EGFP or EGFP-PRAΔTM transfection did not precipitate β-catenin, but EGFP-PRA1 precipitated β-catenin in cells that were both treated or not treated with LiCl (Fig. 3D). If TCF/β-catenin signaling was activated by LiCl, PRA1–β-catenin interaction was much stronger. Therefore, these data show that the up-regulation of PRA1 in the Golgi complex is able to

inhibit the nuclear translocation of β-catenin for TCF/β-catenin signaling by strongly forming PRA1–β-catenin complex, indicating that PRA1 may exhibit diverse functions such as the modulation of cell proliferation and differentiation.

PRA1 is involved in the dephosphorylation of ERK1/2

Wnt signaling has been shown to be involved in extracellular signal-regulated kinase (ERK) pathway activation through β-catenin [24], and Ras-ERK pathway activation has also been known to play a crucial role in cellular proliferation and differentiation. To know whether the ERK1/2-mediated signals are modulated in cells overexpressing PRA1, we examined the ERK phosphorylation and TCF signaling. After HEK293 cells were transfected with PRA1, ERK activation was evaluated in the cell lysates by using Western blotting. The phosphorylated ERK1/2 levels were reduced substantially in the PRA1-cells, as compared to those in the vector controls (Fig. 4A). PRA1 protein in mammalian cell lines, including HEK293 and HCT116 cells, was expressed in weakly detectable levels in our study. Cyclin D1 expression, which is known to be upregulated by TCF/β-catenin signaling, was also repressed in PRA1-cells. The effect of ERK phosphorylation on the TCF/β-catenin signaling was further evaluated via treatment with several protein kinase inhibitors in cell culture. The ERK1/2 inhibitor, PD98059, exerted a profound inhibitory effect on TCF/β-catenin signaling (Fig. 4B). LY294002 and SB203580 also resulted in a moderate inhibition of the signal, whereas the AKT/protein kinase B (PKB) inhibitor produced no such effect. Taken together, the mitogen-activated protein kinases are involved in TCF/β-catenin signaling, and the dephosphorylated ERK in PRA1-cells may negatively affect the β-catenin-involved signal transduction.

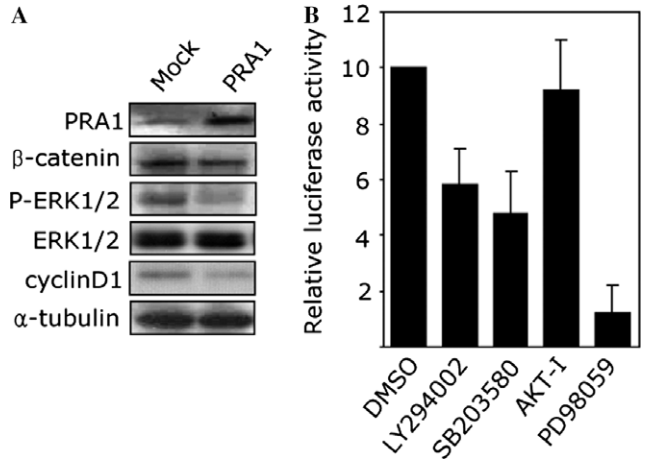

Fig. 4. PRA1 is involved in the dephosphorylation of ERK1/2. (A) Cells were transfected with pcDNA3 or pcDNA3-PRA1, and cell lysates were subjected to SDS–PAGE for Western blotting. In the PRA1-expressing cells, ERK1/2 was dephosphorylated and cyclin D1 was also downregulated. β-Catenin was expressed at similar levels and total ERK1/2 and α-tubulin proteins were used as internal controls. (B) Cells were transfected with TOPflash and treated with various kinase inhibitors, such as LY294002 for PI3K, AKT inhibitor, PD98059 for ERK1/2, and SB203580 for p38 kinase (20 μM) for 1 h.

PRA1 acts to block the TCF/β-catenin signaling in colon cancer cells

In various cancers, including melanomas and colorectal cancers, TCF/β-catenin signaling has been shown to be abnormal, and some mutations of β-catenin and APC are reported in colon cancers [25,26]. Because of this, we examined whether PRA1 affects the TCF/β-catenin signaling in HCT116 colon cancer cells that harbor an oncogenic mutation in β-catenin. Exogenous expression of either wild-type β-catenin or β-cateninΔ1-47, a deletion mutant of the GSK3β-mediated phosphorylation sites, increased the TCF activity as previously shown in HEK293 cells (Fig. 5A). The transfection of N-terminally deleted β-cateninΔ1-47 showed slightly higher activity than that of

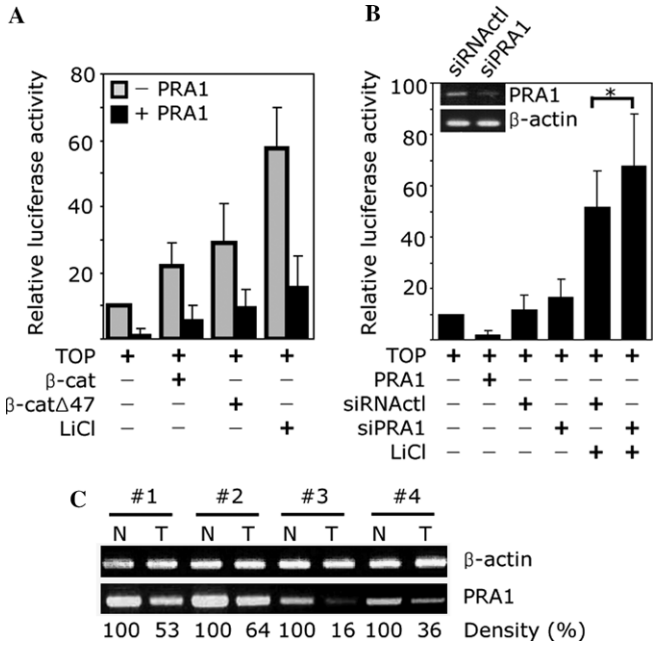

Fig. 5. PRA1 is a strong inhibitor of TCF/β-catenin signaling in colon cancer cells. (A) HCT116 cells were transfected with β-catenin (or β-cateninΔ1-47) in the absence or presence of PRA1, and assayed for TCF activity. Also, cells were treated with LiCl (20 mM for 6 h) and assayed at the same time. (B) HCT116 cells were transfected with plasmids and (or) siRNA for 4 days, and cell lysates were analyzed for TCF activity and used for RT-PCR. The siRNA specific for PRA1 effectively repressed the expression of mRNA (inset). siRNActl indicates a negative siRNA control from manufacturer and β-actin was used as a reaction control. Data are represented as means ± SE from three independent experiments. * $p < 0.005$. (C) Four colon tissue pairs consisting of normal and tumor tissue were prepared, and RT-PCR using their RNA was performed for PRA1 or β-actin. N and T of a tissue pair mean the healthy normal colon and colon tumor from one individual, respectively. Density (ratio of PRA1 to beta-actin) of PCR product in tumor tissue (T) represents relative value to density in normal tissue (N).

wild-type, and treatment with LiCl, a GSK3β inhibitor, induced a strong activity. However, when PRA1 was co-expressed, these TCF activations were largely repressed, indicating that PRA1 also plays an inhibitory role in TCF/β-catenin signaling pathway in colon cancer cells.

Furthermore, using small interfering RNA (siRNA), we examined again whether PRA1 affects on the TCF/β-catenin signaling of tumor cells. HCT116 cells were transfected with various sets of DNA or siRNA, and the TCF activity in each lysate was determined. As shown in the inset, PRA1 mRNA expression was repressed in siPRA1-transfected cells (Fig. 5B). In siPRA1-cells we observed a slight increase of the TCF activity. Subsequently, when LiCl was added to cells, the activity in siPRA1-transfected cells was increased much more than that in control siRNA-cells. Therefore, these findings indicate that PRA1 is surely involved in TCF/β-catenin signaling and inhibits the signaling through interaction with β-catenin. Finally, to compare PRA1 expression levels in colon cancer tissues, we examined the mRNA level of PRA1 in pairs consisting of normal and tumor tissues by using a RT-PCR method. Interestingly, as shown in Fig. 5C, colon cancer tissues randomly selected from clinically diagnosed patients showed significantly decreased PRA1 mRNA expression, which was less than half of the expression level in normal colon tissues. This suggests that PRA1 functions, such as β-catenin binding, might be suppressed by unknown mechanism that exists in colon cancers, and that PRA1 may be important for functioning in tumor prevention and anti-tumorigenesis.

Discussion

PRA1 is a transmembrane protein that initially inserts into the ER compartment, followed by vesicular transport along the exocytic pathway to the Golgi complex where it may function. PRA1 is required for vesicle trafficking from the Golgi complex, and recruits Rab effectors and SNARE [soluble *N*-ethylmaleimide-sensitive factor attachment protein (SNAP) receptor] proteins during this vesicle trafficking process. The cellular localization of PRA1 in the ER and Golgi complex has been reported by several laboratories by using a variety of site-directed or deletion mutants of PRA1 [1,2,12,27]. The C-terminus of PRA1 is a requirement for the Golgi localization and binding to the Rab GTPases, and PRA1 harbors the di-acidic motif DXEE at its C-terminus. The mutation of any one of the acidic residues within this motif results in significant retention in the ER compartment [20]. However, it has been also reported that the deletion of the C-terminal 10 amino acids of PRA1 results in the blockage of its export from the ER, and this di-acidic amino acid substitution exerts no effects on the export of PRA1 from the ER [28]. Alanine-scanning mutagenesis of the entire C-terminal region has revealed that the C-terminal 185Val residue is required for the ER export of PRA1. However, the deletion of 185Val has shown only a moderate effect on the ER export, suggesting that this Val is not involved in export signal itself; rather, it may affect the folding or conformation of PRA1. Furthermore, ER-localized PRA1 mutants or Golgi complex-localized PRA1 wt or mutants have been shown to interact with VAMP2 and Rab. In this study, we have generated a series of ER- or Golgi complex-localized mutants of PRA1, including Golgi-localized PRA1 wild-type, ER-localized PRA-V185E, PRA-DGAA, and ER-Golgi-localized PRAΔ^{185}V. We also generated PRAΔTM (Δ65-164), which is distributed throughout the entirety of the cell (Fig. 3A). Interestingly, the ER- or Golgi complex-localized wild-type or mutant PRA1 variants showed similar results with regard to the inhibition of TCF/β-catenin signaling. However, PRAΔTM did not effectively inhibit the signaling, suggesting that the TM domain of PRA1 is necessary for efficient signaling inhibition. Therefore, our findings indicate that PRA1 functions in the ER, Golgi complex, or ER-Golgi-related vesicles in its membrane-bound conformation.

TCF/β-catenin complex is a transcription factor that is able to modulate multiple genes involved in development and proliferation. β-catenin is also a multifunctional protein that contributes to cell–cell adhesion by strengthening the linkage of E-cadherin and β-catenin to the actin cytoskeleton. It appears to be involved in the Wingless/Wnt signaling pathway, the inappropriate reactivation of which results in tumorigenesis and several biogenic defects. The intracellular concentration and localization of β-catenin are regulated by its degradation, which occurs via interaction with Adenomatous polyposis coli (APC) tumor suppressor protein and phosphorylation at its N-terminus through the interaction with GSK-3β [22,26,29]. Mutations of APC or β-catenin and activation of the Wnt signal inhibit GSK-3β activity and induce β-catenin stabilization [25]. In such a case, β-catenin binds to TCF and the β-catenin-TCF complex translocates into the nucleus, functions as a transcriptional factor, and stimulates tumor formation via increases in the activity of c-myc and cyclin D1 [30]. Interestingly, we demonstrated that the inhibition of TCF/β-catenin signaling by PRA1 does not result in the degradation of β-catenin, nor does it induce the phosphorylation of GSK3β (Fig. 2D). However, importantly, the translocation of β-catenin into the nucleus was markedly reduced by PRA1, and in the Golgi complex, β-catenin was shown to colocalize with PRA1. Also, when we examined the interaction between β-catenin and PRA1 by immunoprecipitation assay, β-catenin was precipitated together with PRA1 in the absence or presence of LiCl (Fig. 3C and D). This observation demonstrates that β-catenin-mediated signals may be regulated by protein-interaction with PRA1, although exact role of PRA1 should be further studied with relation to Rab GTPases.

A number of signals, including TCF/β-catenin signaling, are propagated via the activation of MAPK cascades, which are regulated, in part, by upstream small GTP-binding proteins. Like Ras, Rho GTPases such as Rac1 and Cdc42 are molecular switches that regulate many essential

cellular processes, including cell adhesion, cell cycle progression, and transcription. Cdc42 is involved in c-Jun N-terminal kinase (JNK) activation by many stimuli [31] and cooperates with Raf to activate the ERK pathway. Ras associates with Raf kinase in a GTP-dependent manner, and this complex phosphorylates and activates MAPK/ERK kinase (MEK), and MEK then activates ERK1/2 kinases [32,33]. Therefore, the Ras-ERK pathway is employed ubiquitously in the transduction of cell-type specific growth and differentiation signals from various kinases and receptors. In this study, we have shown that ERK is strongly dephosphorylated in PRA1-cells and similarly, TCF/β-catenin activity is shown to be specifically repressed by ERK inhibitor. Therefore, our results support the notion that mitogen-activated protein kinases are involved in TCF/β-catenin signaling, and the dephosphorylated ERK in PRA1-cells negatively affects the β-catenin-involved signal transduction. However, TCF/β-catenin signaling may also be associated with various signaling networks in cell proliferation and development, such as NF-κB or AP-1 activity. In fact, when we investigated whether NF-κB or AP-1 activity is modulated in the PRA1-cells, PRA1 expression appeared to weakly affect both activities, although basal activity was relatively low in the HEK293 cells used in our study (data not shown). Thus, whether any of the transcriptional regulations are related to PRA1 overexpression and mostly contribute to the PRA1-mediated inhibition of TCF/β-catenin signaling needs further investigation.

Finally, our results have shown that TCF/β-catenin signaling, irrespective of signaling inducers such as exogenous β-catenin and LiCl, is also inhibited in colon tumor cells transfected with PRA1. In contrast, TCF/β-catenin signaling was, to a certain degree, increased in the experiments using siRNA specific for PRA1 in the same tumor cells, although the enhancement of TCF/β-catenin signaling by PRA1 siRNA was not strong compared to its inhibition by PRA1 overexpression. It could be possible that siRNA treatment might be not enough to completely inhibit a low level of endogenous PRA1 protein present in tumor cells. It could also be possible that the PRA1 overexpression effect might be modulated by other proteins such as Rab or VAMP2, and that the inhibitory effect of siRNA on PRA1 function might, therefore, be minimal. In correlation with these findings, PRA1 mRNA level was significantly decreased in primary colon cancer tissues in comparison to normal tissues in the same patients. These findings indicate an interesting possibility that PRA1 expression might regulate tumor development by a modulation of TCF/β-catenin signaling, which is involved in both cellular proliferation and tumor progression, especially in the progression of colon tumor. However, how β-catenin or APC mutations usually observed in colon cancers are related to the PRA1-mediated down-regulation of TCF/β-catenin signaling remains to be determined.

In summary, we demonstrated that PRA1 is crucial for TCF/β-catenin signaling, and that its TCF/β-catenin signal regulation is mediated by an interaction with β-catenin, although in this study PRA1 interaction with other proteins including Rab GTPases has not been investigated in depth. Thus, further studies will be required in order to gain further insight into the role of PRA1 in membranous organelle-mediated signal transduction, and to clarify and elucidate the mechanisms underlying PRA1-mediated inhibition of TCF/β-catenin signaling in the tumorigenesis of colorectal cells.

Acknowledgments

This work was supported by a grant from the Research Center for Women's Diseases of the KOSEF (R11-2005-017-03001) and by the Sookmyung Women's University Research Grants 2006.

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