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RESEARCH ARTICLE

Identification of proteins interacting with the catalytic subunit of PP2A by proteomics

Won-Jeong Lee, Dong-Uk Kim, Mi-Young Lee and Kang-Yell Choi

National Laboratory of Molecular Complex Control, Department of Biotechnology, College of Engineering and Protein Network Research Center, Yonsei University, Seoul, Korea

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The protein phosphatase 2A (PP2A) is a serine/threonine phosphatase involved in the regulation of multiple signaling pathways including the Wnt/β-catenin and the ERK pathways. To understand the complex signaling networking associated with PP2A, we searched proteins interacting with the catalytic subunit of protein phosphatase 2A (PP2Ac) by a pull-down analysis followed by 2-D gel electrophoresis and proteomic analyses. The probability of identification of the proteins interacting with PP2Ac was increased by searching proteins differently interacting with PP2Ac according to stimulation of Wnt3a, which regulates both the Wnt/β-catenin and the ERK pathways. Around 100 proteins, pulled-down by His-tagged PP2Ac, were identified in 2-D gels stained with CBB. By MALDI-TOF-MS analyses of 45 protein spots, we identified several proteins that were previously known to interact with PP2A, such as Axin and CaMK IV. In addition, we also identified many proteins that potentially interact with PP2Ac. The interactions of several candidate proteins, such as tuberous sclerosis complex 2, RhoB, R-Ras, and Nm23H2, with PP2Ac, were confirmed by *in vitro* binding analyses and/or coimmunoprecipitation experiments.

Keywords:
Crosstalk / Interaction / Protein phosphatase 2A

1 Introduction

Protein phosphatase 2A (PP2A) is a major cytoplasmic serine/threonine phosphatase and has a wide range of substrates involving many cellular processes such as growth control [1, 2]. PP2A protein exists as a heterotrimer consisting of a 36 kDa catalytic subunit of protein phosphatase 2A (PP2Ac), a 65 kDa regulatory "A" subunit, and one of several regulatory B-type subunits [1, 3, 4]. PP2A forms stable complexes with cellular signaling molecules such as cytoskeletal and/or structural proteins, and modulates the phosphorylation state and function of the associated proteins [3, 5, 6]. This indicates that the assembly of protein complexes is a general mechanism for functional regulation of PP2A.

PP2A is involved in the regulation of several different signaling pathways such as the ERK and the Wnt/β-catenin signaling pathways involving regulation of cellular proliferation [7–11]. The canonical Wnt/β-catenin signaling is initiated by Wnt ligands, and regulation of the formation of the protein complex containing adenomatous polyposis coli (APC), β-catenin, Axin, and GSK3β is a key event in signal transduction [12, 13]. The ERK pathway is activated by extracellular stimuli such as growth factor, and Ras is an important intermediate signaling molecule in the growth factor mediated activation of the Raf-1→MEK→ERK cascade [14]. However, several scaffold proteins that are known to interact with these signaling components, including SUR8, KSR, and MP-1, have been identified [15–18]. The scaffold proteins are known to be involved in signaling specificity and optimal physiological outputs involving the ERK pathway [19]. PP2A regulates the Wnt/β-catenin signaling pathways by interact-

Correspondence: Professor Kang-Yell Choi, Department of Biotechnology, College of Engineering, Yonsei University, Seoul 120-752, Korea
E-mail: kychoi@yonsei.ac.kr
Fax: +82-2-362-7265

Abbreviations: Con-CM, control-conditioned medium; **His-PP2Ac,** His-tagged PP2Ac; **PP2A,** protein phosphatase 2A; **PP2Ac,** catalytic subunit of protein phosphatase 2A; **TSC2,** tuberous sclerosis complex 2; **Wnt3a-CM,** Wnt3a-conditioned medium

ing with APC and Axin [9, 11, 20–22]. In addition, PP2A interacts with signaling molecules of the ERK pathway such as the receptor tyrosine kinase (RTK), receptor-associated proteins, and all three kinases of the ERK pathway, Raf-1, MEK, and ERK [7, 23, 24]. Because PP2A is known to interact with signaling molecules of the Wnt/β-catenin and ERK pathways, PP2A is an ideal target to investigate complex signaling crosstalk between the two pathways. Using a purified His-tagged PP2Ac (His-PP2Ac) as a bait protein, we searched proteins pulled-down together with His-PP2Ac. Here, we tried to identify proteins interacting differentially with PP2Ac according to physiological signals in order to minimize the possibility of fishing out artifact-binding molecules. We chose Wnt3a as a stimulus in our approach to identify proteins that change interactions with PP2Ac, because Wnt3a is one of the Wnt ligands that activate both the Wnt/β-catenin and the ERK pathways related to growth regulation [25–27].

We used NIH3T3 cells treated with Wnt3a-conditioned medium (Wnt3a-CM) and control-conditioned medium (Con-CM), respectively [25], in preparation of lysates used for pull-down of His-PP2Ac-interacting proteins. By a 2-D gel electrophoresis analysis, we identified proteins that potentially interact with PP2Ac. We also identified several of the proteins previously known to interact with PP2Ac such as Axin [21, 28] and CaMK IV [29, 30]. Many of the newly identified potential PP2Ac-binding proteins were known to be involved in growth control. PP2Ac-bindings to some of the candidate proteins, such as tuberous sclerosis complex 2 (TSC2), RhoB, R-Ras, and Nm23H2, were confirmed by *in vitro* binding analyses and/or coimmunoprecipitation experiments. Further characterization of the PP2A-interacting proteins related to the regulation of the Wnt/β-catenin and the ERK pathways might provide important clues to understand the complex signaling network between these two pathways.

2 Materials and methods

2.1 Vector construction and protein overexpression

A cDNA fragment of PP2Ac was subcloned into the *Bam*HI and *Hind*III site of pRSETB (Invitrogen, Carlsbad, CA), and named pKY-PP2Ac. pGEX-Rheb and pGEX-Nm23H2 were provided by Dr. K. L. Guan (University of Michigan, MI) [31] and Dr. S. K. Chae (PaiChai University, Taejon, Korea) [32], respectively. These were transformed into BL21 star (DE3) pLysS *Escherichia coli* strain (Invitrogen). The transformed cells were grown, and His-PP2Ac proteins were overexpressed by induction with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 22°C for 22 h as described in a previous study [33]. The GST-Rheb and GST-Nm23H2 were overexpressed by induction with 0.5 mM IPTG at 37°C for 4 h 30 min.

2.2 Purification of proteins

The *E. coli* cell pellets were suspended in lysis buffer (20 mM Tris, 500 mM NaCl, and 10 mM imidazole, pH 7.9 for His-tagged protein and PBS, 1 mM DTT, 1% Triton X-100, 1 mM PMSF, 1 mg/mL lysozyme, and protease inhibitor for GST-tagged proteins), and extracts were made as described previously [33]. Recombinant $(His)_6$-PP2Ac was purified by immobilized metal affinity chromatography (IMAC) on Ni-NTA resin (Qiagen, Hilden, Germany). A 20 mL capacity glass column was packed with Ni-NTA agarose matrix and the column was equilibrated with a 7-column volume of the washing buffer (20 mM Tris, 500 mM NaCl, pH 7.9) containing 20 mM imidazole. A 10 mg of cell lysates was loaded onto an Ni-NTA agarose column. The column was washed with washing buffer, and the $(His)_6$-PP2Ac protein was eluted with washing buffer containing 500 mM imidazole. The GST-Rheb and GST-Nm23H2 were purified by GST-Glutathione Agarose (BD Biosciences, Pharmingen, NJ). The cell lysates were loaded onto a GST-glutathione agarose column, and the column was washed with washing buffer (140 mM NaCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.5). The proteins were eluted with elution buffer (10 mM Glutathione, 50 mM Tris, pH 8.0). Each fraction was collected and analyzed by SDS-PAGE.

2.3 Wnt3a-CM, cell extract preparation, and PP2Ac pull-down analysis

The Con-CM and Wnt3a-CM were prepared as described previously [25]. NIH3T3 cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL of streptomycin in 5% CO_2 at 37°C, and treated with either Con-CM or Wnt3a-CM for 30 min as described previously [25]. The cells were washed with PBS and lysed in cold RIPA buffer (100 mM Tris pH 7.9, 150 mM NaCl, 1% NP-40, 1% Triton X-100, 10 mM NaF, and 10 mM imidazole) containing protease inhibitors (1 μg/mL of aprotinin and pepstatin), 1 mM PMSF, and 1 mM Na_3VO_4 for extract preparation. To minimize binding of nonspecific proteins, the cell extracts were precleared with Ni-NTA agarose bead. 400 μg of purified His-PP2Ac was incubated with 400 μL of 50% w/v Ni-NTA agarose bead for 2 h, and then added to the cell extracts and incubated for 2 h at 4°C. The agarose bead was washed twice with RIPA buffer containing 50 mM imidazole, and once more with RIPA buffer without NaCl. The His-PP2Ac pulled-down beads were suspended in 130 μL of rehydration buffer consisting of 7 M urea, 2 M thiourea, 4% w/v CHAPS, 0.4% w/v DTT, 0.5% carrier ampholytes, 40 mM Tris, and 0.001% w/v Bromophenol blue. Proteins were recovered by centrifugation and protein concentrations were determined by the Bradford method.

2.4 2-DE

IEF was performed using a Multiphor II electrophoresis unit (Amersham Biosciences, San Francisco, CA) with 7 cm IPG strips (pH 3–10) according to the manufacturer's instruction. The strips were equilibrated in a buffer consisting of 6 M urea, 2% w/v SDS, 0.375 M Tris-HCl, pH 8.8, 20% v/v glycerol, 130 mM DTT, and 40% acrylamide for 20 min prior to SDS-PAGE. SDS-PAGE was performed on 12% gel at a constant current of 15 mA for approximately 3 h.

2.5 Protein visualization and image analysis

The SDS-PAGE gels were stained with CBB G-250. Protein spots on the gels were scanned as digitalized images using a high-resolution scanner (GS-800 Calibrated Imaging Densitometer: BioRad). With the scanned gel images, the spots were analyzed by Melanie III (Geneva Bioinformatics (GeneBio) S. A., Geneva, Switzerland) after picking several landmarks. Some irregular protein spots that appeared or disappeared were manually defined and further analyzed.

2.6 In-gel protein digestion

Protein spots were excised from the CBB G-250-stained gel using a clean scalpel. The gel pieces were destained with buffer consisting of 25 mM ammonium bicarbonate and 50% v/v ACN. They were dried and reswollen in 25 μL of 25 mM ammonium bicarbonate containing 0.1 μg of sequencing-grade trypsin (Promega, Madison, WI) at 37°C for 16 h. The tryptic peptides were extracted by sonication after adding extraction buffer (25 mM ammonium bicarbonate and 50% ACN) at the same volume as the gel digestion volume, and the extracts were dried in a SpeedVac evaporator.

2.7 MALDI-TOF analysis and database searching

Mass spectra were recorded in the positive ion mode with delayed extraction on a PerSeptive Biosystem Voyager-DE STR MALDI-TOF mass spectrophotometer (Applied Biosystems). The ion acceleration voltage was set to 20 kV, and grid voltage was set 65%. The mass spectra were obtained by averaging 200–300 individual laser shots. Calibration was accomplished by using external mass standards and bovine trypsin autodigestion. The peptide masses were matched with the theoretical peptide masses of proteins to all of the mammalian protein sequences in the NCBI database using the ProFound-Peptide Mapping search engine (http://www.unb.br/cbsp/paginiciais/profound.htm).

2.8 Coimmunoprecipitation and pull-down analysis

pcDNA3.1-HisC-PP2Ac was gifted by Dr. P. B. Rothman (Columbia University, New York) [34]. pcDNA3.0-TSC1-HA and pcDNA3.0-TSC2-HA were provided by Dr. K. L. Guan (University of Michigan) [31], and the pEXV-RhoB WT and pEXV-RhoB V14 vectors were obtained from Dr. A. Hall (London, UK). pcDNA3.0-Nm23H2 were gifted by Dr S. K. Chae (Paichai University) [35]. The cells were grown and transfected with appropriate plasmid(s) by using lipofectamine plus reagent (Life Technologies, Grand Island, N Y), and they were treated with Con-CM or Wnt3a-CM as the case required [36]. After transfection of 36 h, the cells were washed twice with cold PBS and lysed in 500 μL of cold lysis buffer (65 mM Tris-HCl, pH 7.4, 154 mM NaCl, 1% NP-40, 2.5% Na-deoxycholate, 1 mM PMSF, 1 mM EDTA, 1 mM $NaVO_4$, and 1 mM NaF) containing protease inhibitors (1 μg/mL of aprotinin and pepstatin) for 20 min on ice. The cells were harvested, and lysates were prepared as described previously [25]. For a coimmunoprecipitation analysis, 400 μg of cell lysates were incubated with 1–2.5 μg of the appropriate primary antibody (anti-PP2Ac (Upstate, Lake Placid, NY), anti-R-Ras (BD Biosciences), anti-TSC2 (Santa Cruz, CA)) for 12 h, and then with 30 μL of 30% protein A or G-agarose for 4 h. Immune complexes were collected by centrifugation at $230 \times g$ for 3 min followed by three washes with lysis buffer. The immune complexes were subjected to Western-blot analysis. Anti-HA (Santa Cruz) or anti-Myc tag antibody (Cell Signaling, Beverly, MA) were used for immunoprecipitation of the tagged proteins. For the pull-down analysis, 500 μg of cell lysates was incubated with 25 μg of purified GST, Rheb-GST, or Nm23H2-GST overnight at 4°C, and 50 μL of 50% w/v glutathione GST agarose (BD Biosciences) was added and incubated for 30 min at 4°C. PP2Ac was detected by the anti-PP2Ac antibody, and the GST, Rheb-GST, and Nm23H2-GST proteins were detected by the anti-GST antibody (Santa Cruz).

3 Results and discussion

3.1 Pull-down with PP2Ac

PP2A is one of four major serine/threonine protein phosphatases involved in proliferation and differentiation of cells [35]. To understand the crosstalk between the Wnt/β-catenin and the ERK signaling pathways related to PP2A, we searched proteins interacting with PP2Ac by a His-PP2Ac pull-down analysis of NIH3T3 cell extracts followed by a proteomics analysis of the interacting proteins. In order to be used as a bait protein, PP2Ac protein was overexpressed as a His-tagged form in *E. coli*, and purified in soluble form by Ni-NTA agarose column. The purified His-PP2Ac protein was around 37 kDa, and its purity was more than 97% (data not shown). We performed the His-PP2Ac pull-down analysis by using both cell extracts stimulated and nonstimulated with Wnt3a-CM, which activate both the Wnt/β-catenin and the ERK pathways [25], to identify potential PP2Ac-binding proteins more selectively. In addition, we stimulated the NIH3T3 cells used for extract preparation with the Wnt3a-CM, only for 30 min. In this way, we expected to identify proteins differentially interacting with PP2A due to their modifications rather than complex postgene transcriptional events by Wnt3a-CM signaling.

ERK activities and level of phospho-ser-9-phosphorylated GSK3β (p-GSK3β; Ser-9) were immediately increased in the NIH3T3 cells stimulated for 30 min with Wnt3a-CM (Supporting Fig. 1) [25, 36], which indicated the functionality of Wnt3a-CM in the activation of both ERK and Wnt/β-catenin pathways.

3.2 Identification of PP2Ac pulled-down proteins by MALDI-TOF

More than 100 protein spots pulled-down by His-PP2Ac were visualized by Coomassie blue staining of the 2-D gels, respectively (Fig. 1). The positions and intensities of the protein spots were mostly changed on the 2-D gels for Con-CM and Wnt3a-CM, and those proteins can represent potential PP2Ac-binding proteins. We also observed some of the protein spots that appeared in both gels in similar intensities (Fig. 1), and those proteins might represent proteins either interacting with PP2Ac regardless of Wnt3a signaling or proteins interacting with His-tag or bead. We analyzed the protein spots by MALDI-MS analysis and compared them with theoretical peptide masses of all of the proteins in the NCBI database. Among the visualized protein spots, 45 major spots (16 and 29 spots were on Con-CM and Wnt3a-CM gel, respectively) were successfully characterized by MALDI-MS analyses (Tables 1 and 2, respectively). We identified several proteins that have been known to interact with PP2A such as Axin [21, 28] and CaMK IV [29, 30] (Tables 1 and 2; Fig. 2A). These results indicate that our approach to identify PP2Ac-interacting proteins was successful.

3.3 PP2Ac interacts with TSC2, RhoB, R-Ras, and Nm23H2

We identified many of the proteins potentially interacting with PP2Ac (see Tables 1 and 2). Representative 2-D images of the potential PP2Ac-interacting proteins characterized by MALDI-TOF MS analyses are shown in Fig. 2B. Several candidate proteins, such as TSC2, RhoB, R-Ras, and Nm23-H2, which were changed in spot intensity and location by Wnt3a signaling (Fig. 2B), were further tested for their PP2Ac-binding abilities. TSC2 is a potential signaling molecule that functions downstream of the PI3K-Akt pathway and involves various physiologies including proliferation of cells [37]. TSC2 constitutes a complex with TSC1 and functions as a tumor suppressor [38]. To confirm the interaction between TSC1/2 and PP2Ac, we overexpressed HA-TSC1 or HA-TSC2 together with His-PP2Ac (Fig. 3A). His-PP2Ac was coimmunoprecipitated with HA-TSC2, but not with HA-TSC1 (Fig. 3A). TSC2-PP2Ac interaction was also shown by coimmunoprecipitation of endogenous TSC2 and PP2Ac proteins (Fig. 3B). In this case, TSC2-PP2Ac interaction was induced by treatment of Wnt3a-CM, which indicates that TSC2 might interact with PP2Ac in a Wnt3a-signaling-dependent manner (Fig. 3B). The pull-down patterns of TSC2 by His-PP2Ac which are analyzed by 2D-gel (Fig. 2B), however, are different from the immunoprecipitation data. The reason for the absence of TSC2 in the Wnt3a-CM 2-D gel is not clear. The protein spots differently appearing on the 2-D gels might represent proteins diversely modified as well as proteins retaining different affinities for His-PP2Ac dependent upon Wnt3a signaling. Therefore, the intensities of protein spots on the 2-D gel might not always be related to the affinity of the protein for the bait protein. Ras homolog enriched in brain (Rheb) is the direct target molecule of TSC2. TSC2 stimulates the GTP hydrolysis of Rheb and inhibits Rheb activity as a GTPase activating protein [39, 40]. Therefore, we further tested whether Rheb interacts with PP2Ac. As shown in Fig. 3C, endogenous PP2Ac was pulled-down with recombinant GST-fused Rheb (Rheb-GST), but not with GST alone. The level of PP2Ac proteins copurified by Rheb-GST was only slightly increased by Wnt3a-CM (Fig. 3C). It is unknown whether Rheb interacts with PP2Ac directly or *via* TSC2.

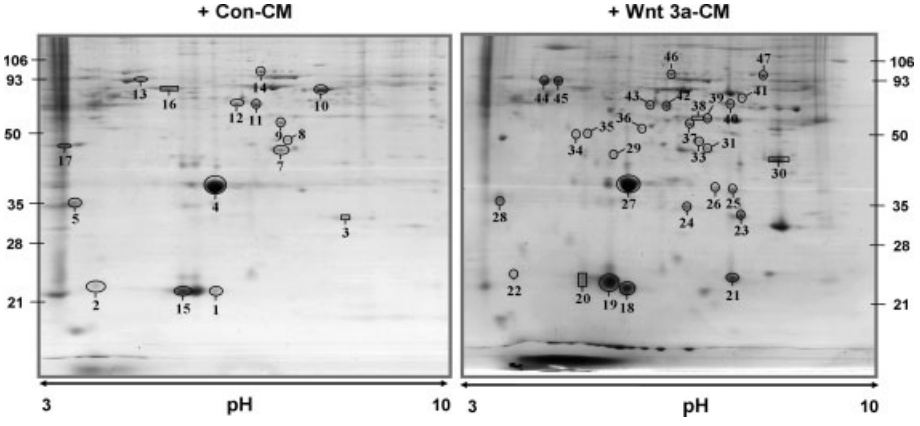

Figure 1. Coomassie-stained 2-D SDS-PAGE gels of proteins pulled-down by PP2Ac. His-PP2Ac-interacting proteins of NIH3T3 cell lysates were prepared as described in Section 2. The pulled-down lysates were subjected to a 7 cm strip, and SDS-PAGE was performed on a 12% gel. The gels were stained with CBB G-250. Left panel: His-PP2Ac-interacting proteins from lysate of NIH3T3 cells treated with Con-CM. Right panel: His-PP2Ac-interacting proteins from lysate of NIH3T3 cells treated with Wnt3a-CM. The numbers represent protein spots characterized by MALDI-TOF analysis.

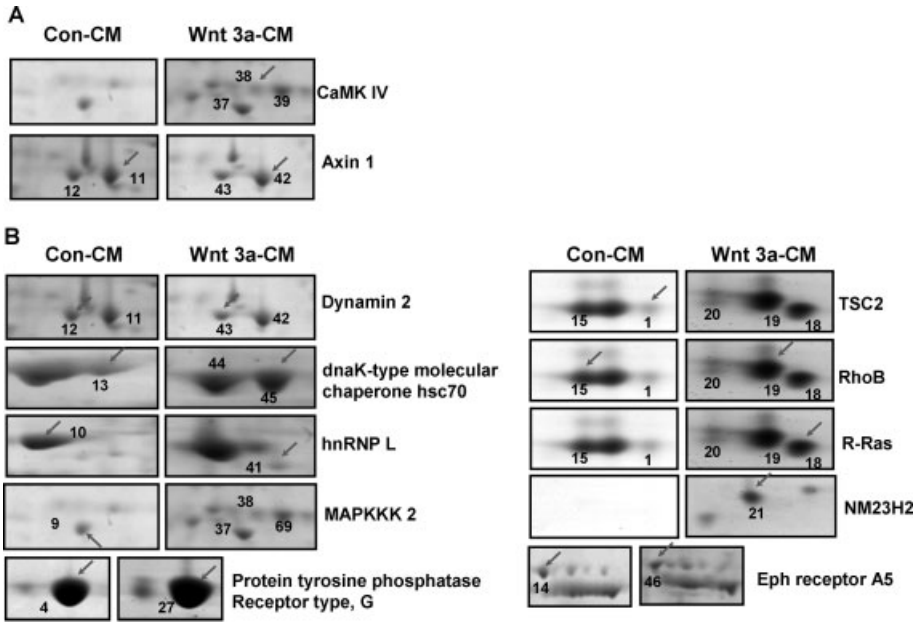

Figure 2. Insets from Fig. 1 showing representative images of altered patterns of PP2Ac-interacting proteins by Wnt3a-CM. For details see Fig. 1. (A) Representative images of the PP2Ac-interacting proteins from lysates of NIH3T3 cells previously known to interact with PP2A. (B) Representative images of PP2Ac-interacting proteins from lysates of NIH3T3 cells potentially interacting with PP2Ac.

Table 1. Identification of PP2Ac-interacting proteins by MALDI-TOF analyses of Con-CM-treated cells

Spot no.	Protein name	Accession no.	MW	p*I*	Coverage %
1	TSC2 (tuberin)	BAA28845.1	22.77	5.9	22
2	Unnamed protein	BAB27979.2	26.78	4.8	18
3	RIKEN cDNA	XP_133893.2	34.8	5.7	26
4	Protein tyrosine phosphatase, receptor type, G	AAH48863.1	32.78	4.8	25
5	Heme-binding protein 2	NP_062360.1	23.12	4.4	16
7	Hypothetical P	XP_157273.1	49.53	7.9	37
8	Mitochondrial ribosomal protein S9	NP_076003.2	45.29	9.2	21
9	Mitogen-activated protein kinase kinase kinase 2	NP_036076.1	70.24	7.8	20
10	Heterogeneous nuclear ribonucleoprotein L	AAH27206.1	60.89	6.7	27
11	Axin 1	AXN1_MOUSE	96.3	6.4	10
12	Dynamin 2	DYN2_MOUSE			
13	DnaK-type molecular chaperone hsc70	A45935	71.1	5.4	26
14	Eph receptor A5	NP_031963.1	98.87	6.2	13
15	RhoB (Ras homolog gene family, member B)	NP_031509.1	22.10	5.1	19
16	RIKEN cDNA	AAH31505.1	81.3	5.5	8
17	Unnamed protein P	BAA95029.1	69.59	5.6	30

The changed protein spots from Fig. 1 were excised and subjected to trypsin digestion followed by MALDI-TOF mass spectroscopy. The data obtained from mass analysis were compared against the NCBI database using the ProFound-Peptide Mapping search engine.

RhoB is one of the Rho family of GTPases that regulates focal adhesion formation, proliferation, motility, transformation, invasion, and transcription [41, 42]. We tested RhoB interaction with PP2Ac by overexpressing Myc-tagged RhoB (RhoB-Myc) in NIH3T3 cells. As shown in Fig. 4A, RhoB-Myc was coimmunoprecipitated with endogenous PP2Ac, and the level of coimmunoprecipitated RhoB-Myc was increased in cells stimulated with Wnt3a-CM. The differential levels of coimmunoprecipitated RhoB-Myc by PP2Ac in cells treated with Con-CM and Wnt3a-CM is not due to differential levels of overexpressed RhoB-Myc (Fig. 4A). PP2Ac was also coimmunoprecipitated with constitutively active RhoB which tagged with Myc (RhoB V14-Myc) without significant coimmunoprecipitation of wild-type RhoB-Myc (Fig. 4B). Again, the differences in the interactions of PP2Ac with RhoB V14-Myc are not due to the different expression levels of RhoB-Myc (Fig. 4B). R-Ras, one of the potential PP2Ac interaction partners, is a small GTPase of the Ras family that regulates cell survival and integrin activity [43]. It is known that R-Ras interacts with several effecter proteins,

Table 2. Identification of PP2Ac-interacting proteins by MALDI-TOF analyses of Wnt3a-CM-treated cells. For details, see Table 1

Spot no.	Protein name	Accession no.	MW	p*I*	Coverage %
18	R-Ras (Harvey rat sarcoma oncogene, subgroup R)	NP_033127.1	23.96	6.3	23
19	RhoB (Ras homolog gene family, member B)	NP_031509.1	22.10	5.1	19
20	RIKEN cDNA	NP_081498.1	21.04	5.8	14
21	Nm23H2 (nucleoside diphosphate kinase 2)	AAH66995.1	17.49	7	32
22	Dermaotontin	NP_062733.1	24.69	4.7	16
23	RIKEN cDNA	XP_133893.2	34.8	5.7	26
24	Hypotetical P	XP_144292.2	34.27	6.2	15
25	γ-actin	CAA31455.1	41.43	5.6	12
26	Protein tyrosine kinase	AAA98465.1	42.28	6.8	12
27	Protein tyrosine phosphatase, receptor type, G	AAH48863.1	32.78	4.8	25
28	Heme-binding protein 2	NP_062360.1	23.12	4.4	16
29	Pyridoxal kinase	AAH27745.1	35.35	5.9	27
30	Hypothetical P	XP_132986.1	46.57	8.9	32
31	Hypothetical P	XP_157273.1	49.53	7.9	37
33	Mitochondrial ribosomal protein S9	NP_076003.2	45.29	9.2	21
34	Polyadenylation element-binding protein 4	NP_080528.1	34.09	5.9	18
35	X-antigen	BAC33089.1	57.42	6.1	12
36	Serine proteinase inhibitor (antitrypsin)	NP_033278.1	47.02	5.6	14
37	Enolase 1, alpha (Rat)	NP_036686.1	70.66	5.9	6
38	CaMK IV	NP_033923	52.63	5.7	8
39	Seryl-aminoacyl-tRNA synthetase	XP_131123.2	61.93	7.2	12
40	Zinc finger protein 69	XP_133298.1	64.9	8.9	17
41	Heterogeneous nuclear ribonucleoprotein L	AAH27206.1	60.89	6.7	13
42	Axin 1	AXN1_MOUSE	96.3	6.4	10
43	Dynamin 2	DYN2_MOUSE			
44	DnaK-type molecular chaperone precursor-mitochondrial	A48127	73.8	5.8	42
45	DnaK-type molecular chaperone hsc70	A45935	71.1	5.4	26
46	Eph receptor A5	NP_031963.1	98.87	6.2	13
47	Hypothetical P	XP_143963.2	84.2	9.7	11

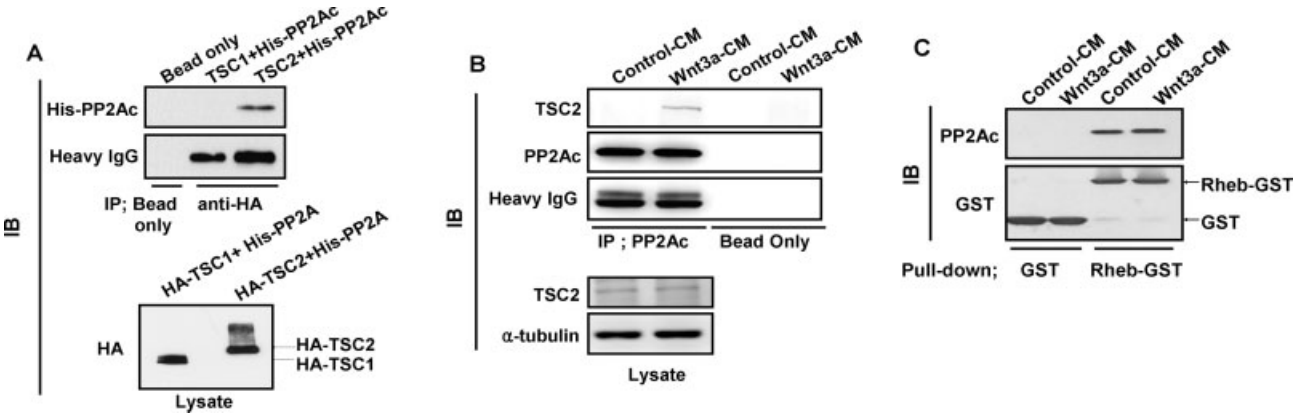

Figure 3. Confirmation of PP2Ac-binding characteristics of TSC2 by *in vitro* binding analyses. (A) NIH3T3 cells were transfected with HA-TSC1 or HA-TSC2 expression vector together with His-PP2Ac vector for 36 h. A 30 μg of lysates was subjected to 10% SDS-PAGE followed by a Western-blot analysis using anti-HA antibody to detect HA-TSC1 and HA-TSC2 (lower panel). Cell lysates were immunoprecipitated with anti-HA antibody, and Western-blot analysis was performed using anti-His mAb to detect His-PP2Ac (upper panel). (B) NIH3T3 cells were grown and treated with Con-CM or Wnt3a-CM for 30 min, and cell lysates were prepared as shown in Fig. 3A. Endogenous PP2Ac was immunoprecipitated by anti-PP2Ac antibody, and coimmunoprecipitated endogenous TSC2 was detected by Western-blot analysis using anti-TSC2 antibody. The lower panel shows the levels of endogenous TSC2 and α-tubulin that were detected by Western-blot analyses using anti-TSC2 and -α-tubulin antibodies, respectively. (C) NIH3T3 cells were treated with Con-CM or Wnt3a-CM for 30 min and cell lysates were prepared as shown in Fig. 3A. The cell lysates were mixed with purified recombinant proteins, GST or GST-Rheb. GST pull-down analyses were performed for both cell lysates stimulated either with Con-CM or Wnt3a-CM using GST-glutathione agarose. Rheb-GST/GST and PP2Ac were detected from the pulled-down complex by Western-blot analyses using anti-PP2Ac and -GST antibodies, respectively.

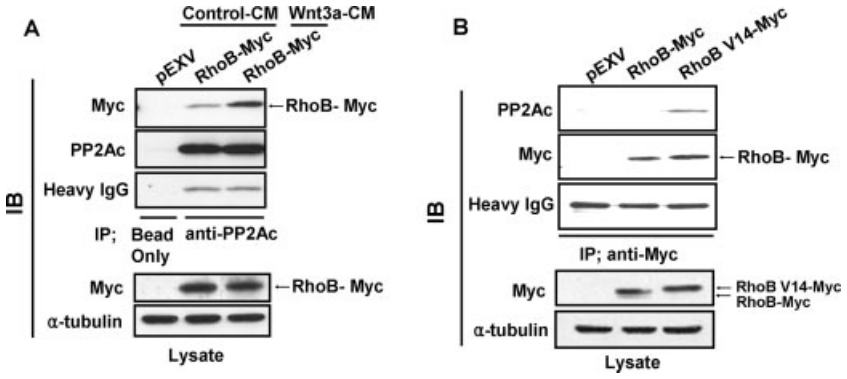

Figure 4. Confirmation of PP2Ac-binding characteristics of RhoB by *in vitro* binding analyses. (A) NIH3T3 cells were transfected with pEXV or RhoB-Myc vector, and treated with Con-CM or Wnt3a-CM as shown in Fig. 3A. RhoB-Myc proteins were coimmunoprecipitated with anti-PP2Ac antibody or with a negative control, bead only, followed by Western-blot analyses using anti-Myc or -PP2Ac antibody. The levels of overexpressed RhoB-Myc were confirmed by Western-blot analysis of the total lysates using anti-Myc antibody. (B) NIH3T3 cells were transfected with pEXV, RhoB-Myc, or RhoB V14-Myc. The cell lysates were immunoprecipitated with anti-Myc antibody, and PP2Ac and RhoB-Myc proteins were detected by Western-blot analyses using anti-PP2Ac or -Myc antibody. The RhoB/RhoB V14-Myc and α-tubulin in the total cell lysates were detected by Western-blot analyses using anti-Myc and -PP2Ac antibodies, respectively.

including c-Raf, the p110 subunit of PI3 kinase, and RalGDS, but the interaction of R-Ras with PP2Ac has not been identified [44]. R-Ras and PP2Ac interacted at the level of endogenous protein, and the interaction was enhanced in cells stimulated with Wnt3a-CM (Fig. 5). Nm23H2 has been found to play a role in regulating the cell shape, differentiation, transcription, and apoptosis [45]. The overexpressed Myc-tagged Nm23H2 (Nm23H2-Myc) was coimmunoprecipitated with endogenous PP2Ac (Fig. 6A). The PP2Ac-Nm23H2 interaction was further shown by *in vitro* pull-down of PP2Ac by purified recombinant Nm23H2-GST but not the GST (Fig. 6B).

Overall, we confirmed the interaction of several potential PP2Ac-binding proteins (TSC2, RhoB, R-Ras, and Nm-23H2) with PP2Ac, using *in vitro* binding analyses and/or coimmunoprecipitation experiments. TSC2 has been a negative regulator in the mTOR pathway, and no information related with PP2A interaction is available. However, recent studies have indicated a role of the complex TSC1/TSC2 in the regulation of the Wnt/β-catenin pathway, and those studies showed interactions of TSC1/TSC2 with signaling components of the Wnt/β-catenin pathways including dishevelled GSK-3β and Axin, among others [46–48]. The identification of Axin–PP2Ac interaction (this study and [21, 28]) provides the relevance for our current identification of TSC2 as a physiological binding partner of PP2Ac. The role of RhoB in the regulation of the Wnt/β-catenin pathway was also indicated, although interaction with PP2Ac was not illustrated. The phosphorylation and nuclear localization of GSK3β were promoted by RhoB overexpression, and the level of β-catenin was decreased by overexpression of the PP2A-B subunit [49]. These results indicate the possible role of RhoB in the regulation of Wnt/β-catenin related with PP2A. R-Ras, one of the PP2Ac-interacting proteins, interacts with many of the signaling molecules related to the Ras/ERK and PI3 kinases pathways, such as Raf-1, PI3 kinase, RalGDS, and others [44]. Therefore, R-Ras might also play a role in the regulation of Ras/ERK and PI3 kinase signaling related with PP2A. The function of Nm23H2 was much less characterized than that of its homologous protein Nm23H1. Nm23H1 is known to bind to the kinase suppressor of ras1 (KSR1), an ERK pathway scaffolding protein, and to regulate Raf-1→MEK→ERK [50]. These results indicate the possibility that Nm23H2, like Nm23H1, also regulates the ERK pathway *via* regulation of PP2A.

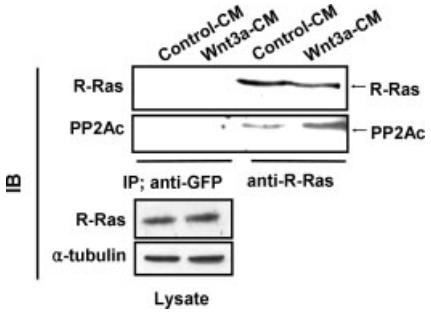

Figure 5. Confirmation of PP2Ac-binding characteristics of R-Ras by *in vitro* binding analyses. NIH3T3 cells were treated with Con-CM or Wnt3a-CM as described in Fig. 3A. The cell lysates were immunoprecipitated with anti-R-Ras antibody, and PP2Ac and R-Ras proteins were detected by Western-blot analyses using anti-PP2Ac or -R-Ras antibody. The two left lanes (Control) represent the immunoprecipitaiton control with anti-GFP antibody. Endogenous R-Ras and α-tubulin proteins were detected by Western-blot analyses using anti-R-Ras and -α-tubulin antibodies, respectively.

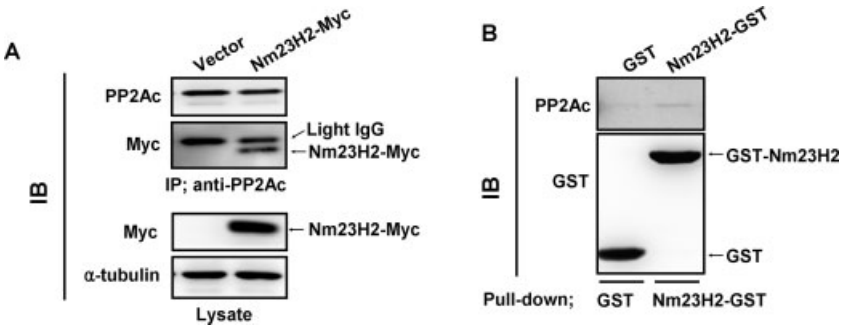

Figure 6. Confirmation of PP2Ac-binding characteristics of Nm23H2 by *in vitro* binding analyses. (A) NIH3T3 cells were transfected with pcDNA3.0 (vector) or pcDNA3.0-Nm23H2-Myc. The cell lysates were immunoprecipitated with anti-PP2Ac followed by Western-blot analyses using anti-Myc or -PP2Ac antibody. (B) NIH3T3 cell lysates were mixed with purified recombinant proteins, GST or Nm23H2-GST, and pulled-down by using GST-glutathione agarose. PP2Ac and Nm23H2-GST/GST were detected from the pulled-down complex by Western-blot analyses using anti-PP2Ac or -GST antibody.

4 Concluding remarks

PP2A regulates multiple signaling pathways involving proliferation of cells, including the Wnt/β-catenin, the ERK, and the PI3 kinase pathways. Therefore, identification and characterization of signaling molecules interacting with PP2A are important in understanding the crosstalk and complicated signaling networks between these signaling pathways related to the control of cell growth. Through this proteomics approach using His-PP2Ac as bait, we identified many of the proteins potentially interacting with PP2A as well as proteins previously known to interact with PP2A. We confirmed the interactions of several candidate proteins with PP2Ac by *in vitro* binding analyses and/or immunoprecipitation experiments. Many of the newly identified PP2Ac-interacting proteins are known to be involved in multiple cellular processes such as cell motility, focal adhesion, metastasis, transformation, and apoptosis. Further characterization of the roles of these proteins in interacting with PP2A will help to elucidate the complex signaling crosstalk related to the control of cell growth.

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5 References

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