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Interaction of the BMPR-IA tumor suppressor with a developmentally relevant splicing factor☆

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Abstract

Inactivation of bone morphogenetic protein signaling via mutation of the BMPR-IA TGF-β superfamily type I receptor causes familial juvenile polyposis, an inherited gastrointestinal cancer predisposition syndrome. In an effort to provide new insight into the mechanism(s) of BMP-mediated tumor suppression, we employed a yeast two-hybrid screen to identify novel proteins that interact with the intracellular domain of BMPR-IA. 30/31 interacting clones encoded SAP49, a splicing factor that has been shown to be required for normal development in *Caenorhabditis elegans*. The remaining interacting clone was FKBP12.6, a known TGF-β type I receptor interactor. The interaction between BMPR-IA and SAP49 was confirmed via coimmunoprecipitation in human cells. Mutational analysis demonstrated that the GS domain of the receptor and the conserved proline-rich domain of SAP49 were required for the interaction. Co-localization studies suggested that the interaction may occur at the inner leaflet of the nuclear membrane. These data suggest that BMPR-IA may interact with and modulate the activity of a developmentally relevant splicing factor.

Keywords: BMPR-IA; Splicing; SAP49

Recent studies have demonstrated that mutational inactivation of bone morphogenetic protein (BMP) signaling pathways is critical to the pathogenesis of sporadic and inherited human cancer. Inherited inactivating mutations of both the BMPR-IA/Alk3 type I receptor and the SMAD4 signaling molecule cause familial juvenile polyposis, an inherited gastrointestinal cancer predisposition syndrome [1–4]. Furthermore, sporadic mutations of SMAD4 are found in a variety of common human tumor types, including adenocarcinoma of the pancreas, colon, and others [5,6].

We have recently provided a variety of functional evidence further pointing to a role for BMP signaling in human cancer. First, we showed that the BMP4 gene is potently transcriptionally regulated by the β-catenin oncogene, which is activated in virtually all sporadic colon cancers [7]. Second, we demonstrated that BMP4 has potent tumor suppressor activity in human cancer cells, and that colon cancer cells are resistant to this tumor suppressive activity [8,9].

BMPs are members of the transforming growth factor-β (TGFβ) superfamily of growth factors, which also includes TGFβs, activins, growth and differentiation factors (GDFs), and the Mullerian inhibiting substance (MIS) or the anti-Mullerian hormone (AMH). BMPs were originally characterized by their ability to induce bone/cartilage formation [10]. However, we now know that BMPs are multifunctional proteins involved in cellular differentiation and ultimately the development of

☆ *Abbreviations:* BMP, bone morphogenetic protein; BMPR-IA, bone morphogenetic protein receptor 1A; BD, binding domain; AD, activation domain; SAP49, splicoseome associated protein 49; TGF-β, transforming growth factor β; ICD, intracellular domain.

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the cardiovascular, nervous, pulmonary, and urogenital systems [11].

BMP ligands initiate signaling by binding to type I and II serine/threonine kinase receptor heterodimers (reviewed in [12,13]). Next, the constitutively active kinase domain of the type II receptor transphosphorylates serine and threonine residues of the type I receptor, predominantly in the conserved glycine/serine rich region (GS domain). This activated type I receptor then phosphorylates receptor-regulated SMADs (R-SMADs: SMAD1, 5, and 8), which then heterodimerize with the common SMAD partner (Co-SMAD) SMAD4, and translocate to the nucleus where they regulate transcription of target genes.

In addition to SMADs, other proteins have been shown to interact with BMP receptors. The SMAD ubiquitin regulatory factor-1 (Smurf1) binds to and inhibits BMPR-IA through its interaction with the inhibitory SMADs SMAD6 and SMAD7 [14]. The integral membrane glycoprotein endoglin binds to TGFβ type I and II receptors, including BMP receptors [15]. LIM kinase 1 (LIMK1) binds to the carboxy-terminal tail of BMPR2 [16]. Kurozumi et al. [17] have shown that BMPR-IA interacts with the BMP receptor-associated molecule 1 (BRAM1). Finally, FKBP12 and FKBP12.6 bind to residues within the GS domain of type I receptors [18,19].

Because of recent evidence of the tumor suppressive properties of BMPR-IA, we decided to search for novel proteins that interact with the intracellular domain of the BMPR-IA receptor.

Materials and methods

Yeast two-hybrid. The Proquest Yeast Two-Hybrid kit with Gateway technology (Invitrogen) was used as described by the manufacturer. Briefly, the intracellular domain of BMPR-IA was amplified out of the hALK3 expression vector (a kind gift of Dr. Peter ten-Dijke) by polymerase chain reaction (PCR). The resulting PCR product was cloned into the Gal4 DNA binding domain yeast expression vector pDEST32. The resulting expression vector was cotransformed into MaV203 competent yeast (Invitrogen) along with either the human fetal brain gateway-compatible Gal4 activation domain cDNA library (Invitrogen) or the human liver Gal4 activation domain cDNA library (Invitrogen). Transformed yeast were selected for on −His −Leu −Trp + 10 mM 3AT plates grown at 30 °C for 72 h. Putative interacting clones were gridded on a master −Leu −Trp plate for further reporter tests. Colonies were resuspended in 100 μl autoclaved saline and diluted 1:10. A 7 μl dot was transferred to −Leu −Trp −Ura, −Leu, −Trp + 5FOA or −His −Leu −Trp + 10 mM 3AT plates, allowed to dry, and incubated at 30 °C for 72 h. In addition, clones were gridded onto nylon membranes placed on YEPD agar plates and incubated at 30 °C for 24 h. The membranes were flash-frozen in liquid nitrogen and placed on filter paper soaked in X-gal solution [10 mg X-gal dissolved in 100 μl DMF mixed with 60 μl of 2-mercaptoethanol and 10 ml filter sterilized Z buffer (16.1 g $Na_2HPO_4 \cdot 7H_2O$, 5.5 g $NaH_2PO_4 \cdot H_2O$, 0.75 g KCl, 0.246 g $MgSO_4 \cdot 7H_2O$ dissolved in 1 L H_2O, pH 7.0)]. The membranes were incubated at 37 °C for 24 h and any color changes were noted.

Clones that successfully passed at least three out of four reporter tests were harvested for DNA using the Yeast RPM Kit (Qbiogene, Carlsbad, CA). AD clones were recovered by selecting on LB agar plates supplemented with ampicillin. Resulting colonies were grown in overnight cultures and plasmid DNA was recovered using the Nucleobond AX Plasmid Maxi kit (BD Biosciences, Palo Alto, CA). The putative interacting AD clones were then cotransformed into MaV203 with the BMPR-IA BD clone and the reporter tests were repeated to confirm the interaction. Successful retransformants were sequenced to identify the insert in the pEXP-AD402 vector representing the putative interacting protein. All yeast media and supplements were purchased from Qbiogene.

Tissue culture. HEK293 and NTERA2 (also known as NT2/D1 or NT2 clone D1) cells were obtained from the American Type Culture Collection (Manassas, VA). All media, supplements, and trypsin were obtained from Invitrogen (Carlsbad, CA). Unless otherwise noted, media were supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. HEK293 cells were grown in minimal essential media (MEM). NTERA2 cells were grown in Dulbecco's modified Eagle's medium (DMEM). BMP4 (R&D Systems, Minneapolis, MN) was reconstituted in sterile PBS and used at a final concentration of 100 ng/ml. Transfections were performed with Lipofectamine or Lipofectamine 2000 reagent (Invitrogen) as directed by the manufacturer. Cells were harvested by adding cell lysis buffer (Cell Signaling Technology, Beverly, MA) supplemented with Complete Mini protease inhibitor cocktail tablets (Roche, Indianapolis, IN), scraping cells, and collecting lysates. Protein concentrations were determined by the BCA protein assay (Pierce, Rockford, IL).

Immunoprecipitation and Western blots. SAP49 expression vectors were generated using the Bidirectional TA cloning kit (Invitrogen) according to the manufacturer's recommendations. Briefly, the coding region of SAP49 was amplified using PCR with Expand polymerase (Roche) and ligated into pCR3.1 (Invitrogen), resulting in SAP49 expression vectors harboring either a C- or N- terminal FLAG epitope tag (DYKDDDDK). HEK293 cells were cotransfected with C- or N- FLAG SAP49 and C-HA hALK3 expression vectors. After 24 h, cells were harvested, and immunoprecipitations were performed using the FLAG immunoprecipitation kit (Sigma, St. Louis, MO). Six hundred micrograms of whole cell lysate was incubated with mouse-α-HA- or mouse-α-FLAG- preconjugated agarose beads (Sigma) for 48 h at 5 °C. The beads were washed three times with lysis buffer and were boiled in 5× gel loading buffer (all provided with the FLAG IP kit). The resulting supernatant was separated by SDS–PAGE on Bis–Tris NUPAGE precast gels (Invitrogen), transferred to PVDF membranes, probed with primary and horseradish-peroxidase coupled secondary antibodies, and visualized by ECL (Amersham, Piscataway, NJ). Mouse-α-HA primary antibody was obtained from Babco, rabbit-α-HA, primary antibody was obtained from Zymed, rabbit-α-FLAG primary antibody was obtained from Sigma, goat-α-mouse secondary antibody was obtained from KPL (Gaithersburg, MD), and donkey-α-rabbit secondary antibody was obtained from Pierce.

Site-directed mutagenesis. The Quikchange XL site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) was used according to the manufacturer. Primers were designed to introduce nonsense mutations in pDEST32-BMPR-IA at amino acid positions 207, 291, and 385, and in pEXP-AD502-SAP49 at amino acid positions 102, 199, 341, 369, and 397. Clones were sequenced to confirm the presence of the desired induced mutation.

Immunofluorescence. NTERA2 cells were seeded on sterile glass coverslips placed inside wells of six-well plates and grown until they reached 80% confluence. Cells were transfected with 1.15 μg C-HA-BMPR-IA (a kind gift of Dr. Peter ten-Dijke) and 1.15 μg of pCR3.1-C-FLAG-SAP49 using Lipofectamine reagent (Invitrogen). Twenty four hours after transfection, cells were fixed in phosphate-buffered saline (PBS) + 3.7% formaldehyde solution and permeabilized with PBS + 0.2% Triton X. Fixed, permeabilized cells were stained with

mouse α-HA (Babco) and rabbit α-FLAG (Sigma) primary antibodies and FITC-conjugated goat α-rabbit and CY3-conjugated goat α-mouse secondary antibodies (Molecular Probes). Coverslips were mounted on slides using Vectashield with DAPI (Vector Laboratories, Burlingame, CA). Cells were visualized with an Olympus IX-70 inverted confocal microscope. Images were assembled and analyzed with Metamorph software (Universal Imaging, Downingtown, PA).

SAP49 expression analysis. To determine the level of SAP49 expression in a variety of human tissues, a human 12 lane multiple tissue Northern blot (Clontech) was probed with a PCR product encoding the human SAP49 cDNA. Briefly, following overnight 68 °C prehybridization in QuikHyb (Stratagene), the membrane was hybridized for 24 h at 68 °C in QuikHyb containing a radiolabeled human SAP49 probe. The membrane was then washed and imaged using a PhosphorImager 445 SI (Amersham Biosciences, Piscataway, NJ).

MSX2 reporter assays. Confluent NTERA2 cells were passaged 1:3 into the wells of a six-well plate. Approximately, 48 h after plating, the cells were cotransfected with 1.28 μg of the MSX2-lux firefly luciferase reporter (generously provided by Christian Sirard, McGill University), 1.28 μg N-FLAG or C-FLAG-SAP49, and 1.28 μg tkRenilla (Promega) as an internal control for transfection efficiency using Lipofectamine 2000 reagent (Invitrogen). 24 h after transfection, the cells were washed once with PBS and harvested in 500 μl of 1× Passive Lysis Buffer (Promega). Firefly luciferase and *Renilla* luciferase activities were measured with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI), as recommended by the manufacturer, using a Microlumat Plus LB 96V (Berthold Technologies, Germany) luminometer. Data were analyzed with Winglow (Berthold Technologies) software.

Results and discussion

BMPR-IA yeast two-hybrid screening

To identify BMPR-IA interacting proteins, the intracellular domain (ICD) of BMPR-IA was used as "bait" in a yeast two-hybrid screen as described in Materials and methods. First, a human fetal brain library was screened. Of the 31 interacting proteins isolated from the human fetal brain library, 30 were identified as splicosome-associated protein subunit 4 (SAP49), also known as splicing factor 3b, subunit 4 (Sf3b4). Of the 30 SAP49 clones, there were 10 *distinct* clones (with different vector/insert junctions). BMPR-IA and SAP49 were shown to interact in each of the four reporter tests available (Fig. 1), indicating a strong level of interaction. The other interacting protein identified from the human fetal brain library was FK506 binding protein (FKBP) 12.6, a protein having 83.3% amino acid homology with the well-known TGFβ type-I receptor interactor FKBP12. FKBP12.6 has also been shown to interact with type I TGFβ receptors through yeast two-hybrid screening [19]. The recovery of a known interactor of type I TGFβ receptors provided valuable internal validation of our yeast two-hybrid screen.

Next, we repeated the yeast two-hybrid screening with a second cDNA library, this time derived from human liver, and constructed in a different vector backbone. Nine interacting proteins were isolated from the human liver library; all were SAP49 (two distinct clones). The identification of SAP49 from two independent libraries provides confirmation of our results. We then repeated the yeast two-hybrid screens using the intracellular domain of the constitutively activated form of BMPR-IA (Q223D) as bait. No interacting clones were identified, suggesting that in the activated state, a conformational change prevents binding of the receptor to interacting proteins such as SAP49.

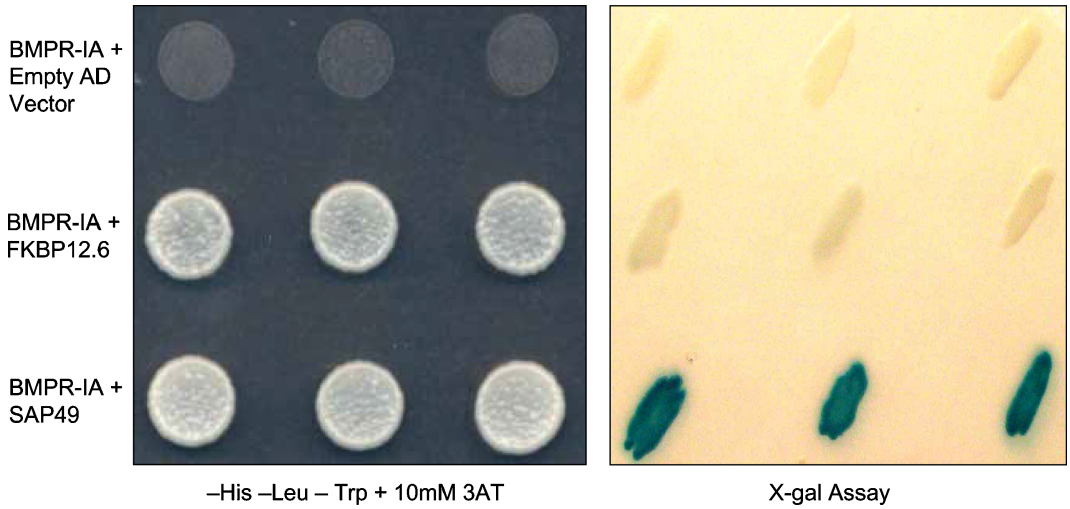

Fig. 1. Interaction of SAP49 and BMPR-IA in *Saccharomyces cerevisiae*. SAP49 interacted strongly with the BMPR-IA intracellular domain, scoring as an interactor in each of the four available two-hybrid reporters. The (−) His auxotrophy and lacZ reporter tests are shown above. Other tests performed were the (−) Ura auxotrophy and the 5FOA negative selection test for (−) Ura auxotrophy (data not shown). The interaction between FKBP12.6 and BMPR-IA was somewhat weaker. Although FKBP12.6 scored as an interactor in the (−) His auxotrophy test, it scored as a weak interactor in the lacZ reporter test shown above.

Full-length BMPR-IA interacts with SAP49 in human cells

To validate the interaction between BMPR-IA and SAP49 in vivo, we generated SAP49 expression vectors with either C- or N-terminal FLAG epitope tags (see Materials and methods). The expression vectors were co-transfected with a full-length BMPR-IA expression vector with a C-terminal HA epitope tag into HEK293 cells and coimmunoprecipitation/Western blot analysis was performed (Fig. 2). When either N-FLAG- or C-FLAG-SAP49 was immunoprecipitated with α-FLAG-conjugated agarose beads, BMPR-IA coimmunoprecipitated (lanes 4 and 5). No interaction was present in cells transfected with one, but not both, expression vectors (lanes 1–3) or in untransfected cells (lane 6). To determine the specificity of SAP49/BMPR-IA interaction, N-FLAG and C-FLAG-SAP49 expression vectors were cotransfected with an unrelated expression vector containing a C-terminal HA epitope tag. N-FLAG and C-FLAG SAP49 failed to interact (data not shown), confirming the specificity of the interaction. Of note, although lane 1 of Fig. 2 shows a weak band in the absence of HA-BMPR-IA, further coimmunoprecipitations confirmed that the faint band was due to inadequate washing of the beads (data not shown).

BMPR-IA and SAP49 localization

The reproducibility and specificity of the BMPR-IA/SAP49 interaction demonstrated by yeast two-hybrid and coimmunoprecipitation provided substantial evidence for the authenticity of the interaction. However, because BMPR-IA is a cell surface receptor and SAP49 is a nuclear protein, further validation of the interaction was needed. Thus, the localization of BMPR-IA and SAP49 was studied to determine if the two proteins are expressed in close enough proximity to interact within an intact cell. As expected for a splicing factor, SAP49 exhibited strong and homogeneous nuclear staining (Fig. 3A) in NTERA2 cells. Surprisingly, BMPR-IA did not show the strong plasma membrane staining expected for a cell surface receptor. Instead, faint BMPR-IA staining was seen throughout the cytoplasm, with strong staining localized in a perinuclear formation (Fig. 3B) possibly staining the nuclear envelope or the rough endoplasmic reticulum. The proximity of the two proteins is consistent with the ability to interact (Fig. 3C).

Characterization of BMPR-IA/SAP49 interaction

To further characterize the interaction between BMPR-IA and SAP49, SAP49 truncation mutants were generated as described in Materials and methods and tested for their ability to interact with the BMPR-IA ICD in a yeast two-hybrid assay (Fig. 4). The carboxy terminal proline-rich domain of SAP49 was required for the interaction. Furthermore, there was a graded degree of interaction; the more SAP49 carboxy terminus was present, the greater the strength of the interaction. It is intriguing that though the RNA binding domains

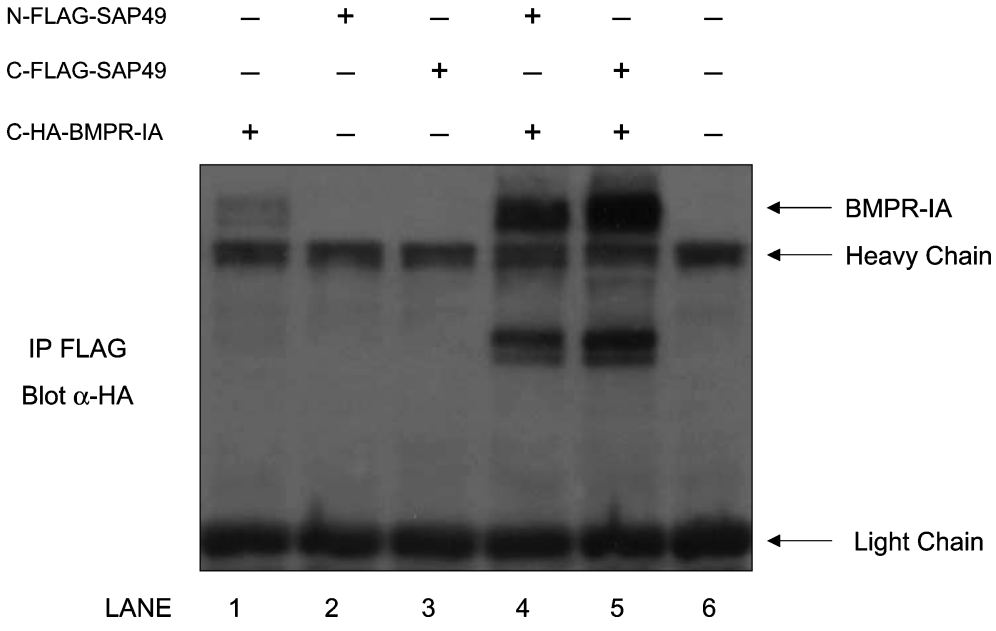

Fig. 2. Interaction of SAP49 and BMPR-IA in human cells. HA-tagged BMPR-IA coimmunoprecipitated with both N- and C-terminal-tagged SAP49 when co-expressed in 293 cells (lanes 4 and 5). No interaction was present in cells transfected with one, but not both, expression vectors (lanes 1–3) or in untransfected cells (lane 6). Heavy and light chain background bands are seen in all lanes. The faint BMPR-IA band in lane 1 is due to incomplete washing of the beads and was not present in replicate experiments.

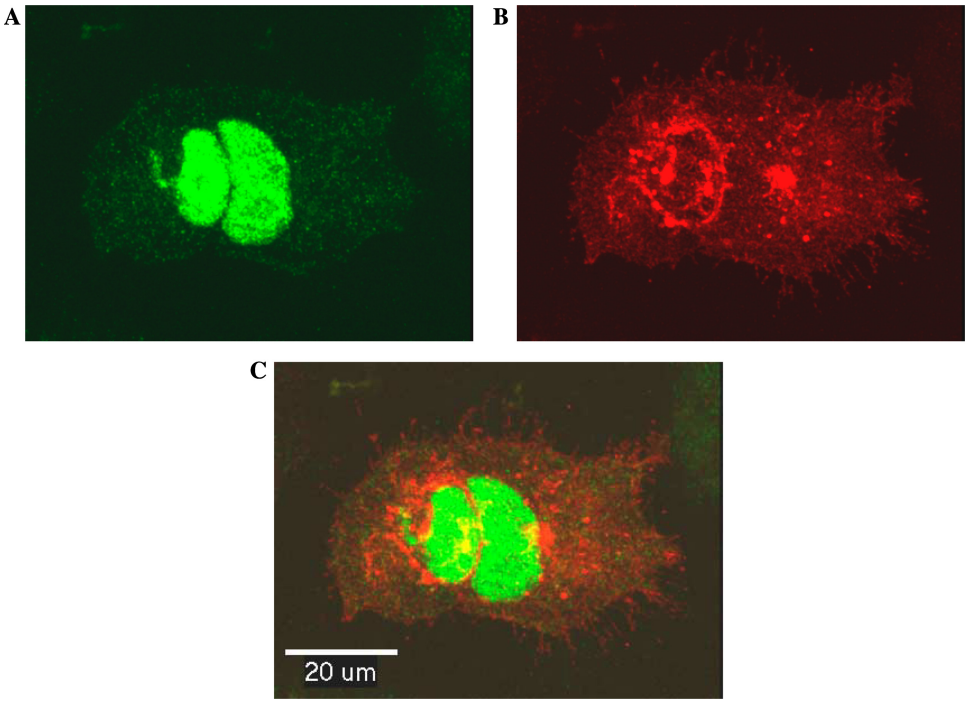

Fig. 3. Co-localization of BMPR-IA and SAP49. N-FLAG-SAP49 and C-HA-BMPR-IA expression vectors were cotransfected in NTERA2 cells and protein localization was studied by immunofluoresence with anti-FLAG and anti-HA antibodies. (A) FITC staining of SAP49 shows nuclear localization. (B) CY3 staining of BMPR-IA shows cytoplasmic staining with a distinct perinuclear ring. (C) Overlay of (A) and (B).

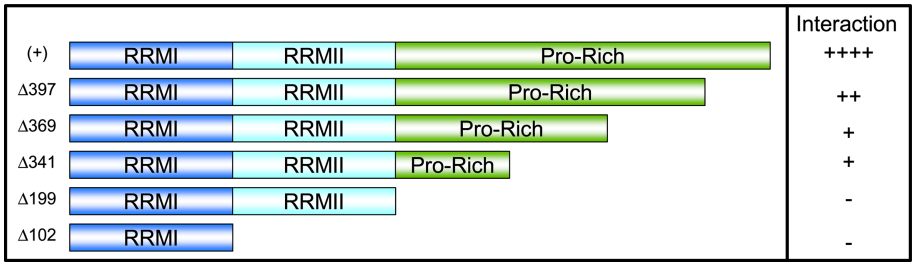

Fig. 4. SAP49 interaction domain. Site-directed mutagenesis was employed to create truncations of SAP49 having only one RNA recognition motif (Δ102), both RNA recognition motifs (Δ199), and various lengths of the proline-rich domain (Δ341, Δ369, and Δ397). Yeast two-hybrid reporter tests of these SAP49 mutants demonstrated that the proline-rich domain was required for interaction with the BMPR-IA ICD. The Δ341, Δ369, and Δ397 mutants, containing increasing lengths of the proline-rich domain, had increasing levels of interaction.

of SAP49 are highly conserved from yeast to higher eukaryotes, the carboxy terminal proline-rich domain is only present in SAP49 from higher multicellular eukaryotes with complex developmental programs such as the human, fly, and worm [20,21]. Taken together, these data indicate that the BMPR-IA ICD interacts with the proline-rich carboxy terminal region of SAP49.

The intracellular domain of BMPR-IA is comprised of the GS domain and a large serine–threonine kinase domain broken down into several sub-kinase motifs. To identify the specific regions of the BMPR-IA ICD required for interaction with SAP49, BMPR-IA truncation mutants were generated (Fig. 5). Interestingly, the most dramatically truncated Δ207 mutant (retaining only the GS domain and the first two sub-kinase domains) retained the ability to partially interact with SAP49, while the Δ291 and Δ385 truncation mutants were unable to interact. These data indicate that the GS domain is sufficient for the interaction with SAP49, and suggest that conformational changes in the partially truncated intracellular domain may mask this interaction.

SAP49 expression analysis

Northern blot analysis with a human 12 tissue multiple tissue Northern blot was performed to examine the tissue distribution of SAP49 expression. As depicted in

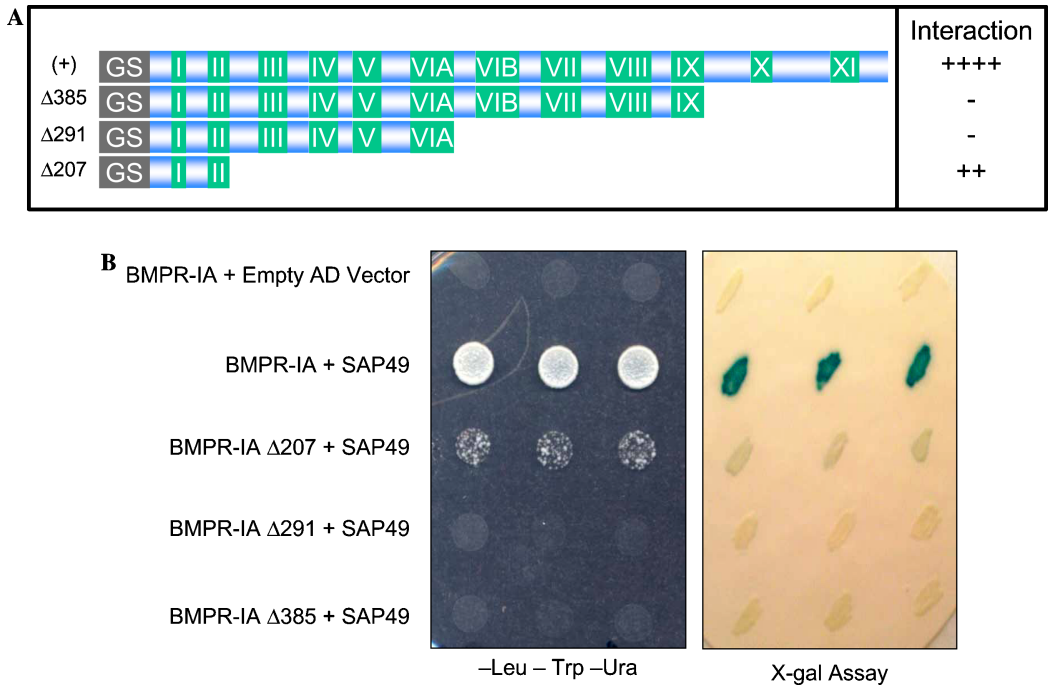

Fig. 5. BMPR-IA interaction domain. (A) Site-directed mutagenesis was employed to create truncations of the BMPR-IA ICD having the GS domain and two kinase subdomains (Δ207), six kinase subdomains (Δ291), and nine kinase subdomains (Δ385). Yeast two-hybrid reporter tests of these BMPR-IA mutants demonstrated that the GS domain was required for interaction with SAP49. (B) Examples of yeast two-hybrid reporter tests of these BMPR-IA mutants. The Δ207 mutant has moderate activation of the reporters, while Δ291 and Δ385 have no activation of the reporters. Other tests performed were the (−) His auxotrophy and the 5FOA negative selection test for (−) Ura auxotrophy (data not shown).

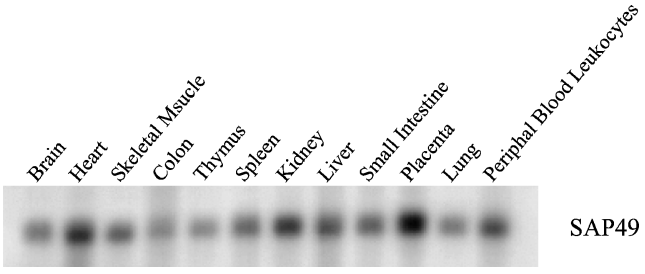

Fig. 6. SAP49 expression analysis. A 12 tissue multiple tissue Northern blot was hybridized with a radiolabeled SAP49 probe. SAP49 expression was the strongest in heart, kidney, and placenta, and weakest in colon, thymus, and lung.

Fig. 6, SAP49 is expressed in virtually all human tissues. SAP49 is most highly expressed in heart, kidney, and placenta.

Functional consequence of BMPR-IA/SAP49 interaction

Unfortunately, there are no readily available assays that would enable us to determine if BMP signaling can modulate SAP49 function. Therefore, we focused our efforts on determining whether SAP49 can modulate BMP signaling. In particular, we studied the effect of SAP49 on the ability of BMP4 to induce a well-known BMP-regulated promoter, that of the MSX-2 homeobox gene. In the absence of SAP49, the MSX-2 reporter was induced 4.3-fold by BMP4 when compared to untreated NTERA2 cells. Co-transfection of SAP49 expression vectors had no effect on reporter activation by BMP4, demonstrating that SAP49 does not modulate the classical SMAD-mediated BMP4 signaling pathways.

SAP49 is a developmentally relevant splicing factor

Pre-mRNA splicing in mammals is catalyzed by two main splicosome complexes, the major (U2-type) and the minor (U12-type) spliceosomes. These spliceosomes differ in their splice site specificity and their subunits, termed small nuclear ribonucleoprotein particles (snRNPs) (reviewed in [22]). SAP49 is a structural component of the SF3B splicing factor that promotes branch point recognition by the U2 snRNP (major) or the U11/U12 di-snRNP (minor) during the splicing reaction. SAP49 is required for proper development of *Caenorhabditis elegans*. When antisense SAP49 is injected into worms, embryos laid by the worms ceased development at the morphogenesis stage of embryogenesis [23].

Interaction between splicing components and cell signaling

Associations between cell signaling components and splicing factors have been described previously. For example, a recent report describes the association of SAP145 (also known as splicing factor 3b, subunit 2) with SMAD3, which transduces activin and TGFβ signals to the nucleus [24]. Interestingly, SAP145 interacts

directly with SAP49 through the SAP49 RNA recognition motifs [25]. Also, cyclin E/cdk2 complexes have been shown to bind to several spliceosome associated proteins, including SAP145 and SAP155 [26].

Effects of BMPR-IA on SAP49 function

We were unable to detect any effect of SAP49 on BMP4 signaling. However, we are intrigued by the possibility that the interaction between BMPR-IA and SAP49 may regulate the splicing activity of SAP49. Since both BMP4 and SAP49 appear to be important for normal development, we hypothesize that their interaction may play a role in regulating tissue-specific and/or developmental-stage specific splicing. Further studies to determine the functional consequences of this novel interaction appear warranted.

Acknowledgments

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