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TSAd interacts with Smad2 and Smad3

K.C. Richard [a], G.E. Bertolesi [a,1], L.D. Dunfield [a,2], C.R. McMaster [b], M.W. Nachtigal [a,c,*]

[a] Department of Pharmacology, Dalhousie University, 5850 College Street, Halifax, NS, Canada B3H 1X5
[b] Departments of Pediatrics and Biochemistry and Molecular Biology, Dalhousie University, Halifax, NS, Canada B3H 1X5
[c] Department of Medicine, Dalhousie University, Halifax, NS, Canada B3H 1X5

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Abstract

Smad-dependent signalling initiated by TGFβ superfamily members can be modulated by a variety of interacting proteins. Using yeast two-hybrid, co-immunoprecipitation, and GST pull-down assays we identified T-cell SH2 adapter (TSAd) as a protein that interacts with Smad2 and Smad3. TSAd is an adapter protein thought to participate in many different signalling pathways. The objective of this study was to elucidate the domains important for interaction between TSAd and Smad proteins. Our results suggest a model for TSAd–Smad interaction that is facilitated by multiple TSAd domains, but primarily through the TSAd type I SH2 domain. Interestingly, we also found that both Smad2 and Smad3 interact with the Lck type I SH2 domain, but not the PI3K type III SH2 domain. This research raises the possibility that interaction between SH2-containing proteins and Smad proteins may represent another method to modulate Smad-dependent signalling.

Keywords: Smad2; Smad3; TSAd; Lad; SH2 domain; GST-pull-down; Co-immunoprecipitation

Transforming growth factor β (TGFβ) superfamily signalling is partially mediated through the intracellular Smad proteins [1,2]. For example, TGFβ signalling is initiated by ligand binding to type II receptors resulting in the recruitment and transphosphorylation of the TGFβ type I receptor. Both receptors are dimeric transmembrane serine/threonine kinases that form an activated tetramer upon ligand binding. Intracellular signalling is mediated by phosphorylation of the receptor-activated Smad proteins, Smad2 and Smad3, which subsequently form a complex with Smad4. Heterodimeric or trimeric Smad complexes translocate to the nucleus and associate with other transcription factors and co-factors to modulate the expression of TGFβ-responsive genes.

Numerous Smad adapter proteins have been identified that participate in cell-specific TGFβ signalling activities [3–7]. Using a yeast two-hybrid screen designed to identify Smad2-interacting factors, we identified a novel interaction between Smad2 and the T-cell SH2 domain adapter protein (TSAd). The TSAd protein has been recently described as an intracellular adapter protein in the T-cell receptor (TCR) signalling pathway. This protein was identified in separate yeast two-hybrid screens (human and mouse) as a protein which interacts with Src family kinases (such as Lck) and Tec-family kinases (such as RLK and ITK) [8–10]. It was also identified as a protein that interacted with the vascular endothelial growth factor (VEGF) receptor, and called VEGF receptor-associated protein (VRAP) [11]. The mouse orthologue is called Lad (Lck-associated adapter protein) or RIBP (RLK/ITK binding protein), and these proteins are transcribed from the *SH2D2A* gene [12]. TSAd was initially cloned from human hematopoietic cells and its expression was thought to be restricted to T

* Corresponding author. Fax: +1 902 494 1388.
E-mail address: Mark.Nachtigal@Dal.Ca (M.W. Nachtigal).
[1] Present address: University of Calgary, 3330 Hospital Drive NW, Calgary, Alta., Canada T2N 4N1.
[2] Present address: Canadian Agency for Drugs and Technologies in Health (CADTH), 600-865 Carling Avenue, Ottawa, Ont., Canada K1S 5S8.

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cells, where it acts as a modulator of T-cell receptor signalling [8]. However, TSAd is also expressed in lung epithelial cells where it interacts with a variety of intracellular signalling molecules in response to PDGF or EGF treatment [13]. Human TSAd possesses typical adapter protein motifs including a SH2 domain, a putative proline-rich SH3-binding domain, and several potential tyrosine phosphorylation sites [8,9,14]. The mechanisms underlying receptor regulated protein–protein interaction have not been clarified, although TSAd appears to play a regulatory role in several signal transduction pathways that affects the expression of downstream target genes [9–11,13–15].

The objective of this study was to elucidate the interaction between TSAd and Smad proteins. The interaction between TSAd and Smad2 was confirmed using GST pull-down and co-immunoprecipitation (co-IP) experiments. We have also determined that TSAd can also interact with Smad3, but not Smad4. Using GST pull-down methods, we have further elucidated the interacting domains between the TSAd and Smad proteins. Our results suggest that TSAd–Smad interaction is facilitated by sites in the amino (N) and carboxy (C) terminal portions of the TSAd protein, but primarily through the TSAd type I SH2 domain.

Materials and methods

Yeast two-hybrid assay. PCR primers were designed to allow directional subcloning of full-length human Smad2 cDNA into the pAS1.-CYH2 vector (BD Biosciences Clontech, Palo Alto, CA) to generate the yeast expression vector pAS1.CYH2-Smad2. pAS1.CYH2-Smad2 was transformed into yeast strain PJ69 (ATCC 201450). Procedures to screen a human lymphocyte cDNA library in pACT and verify positive interactions were conducted according to the procedure outlined in [16].

Cell transfection and GST pull-down. COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; CanSera, Etobicoke, Ont.). COS-7 cells were transiently transfected with full-length Smad2 and Smad3 expression vectors (obtained from Dr. L. Attisano, University of Toronto; [17,18]) and N- or C-terminal portions of Smad proteins (obtained from Dr. K. Luo, Lawrence Berkeley Labs; [19]). Cells were transfected with 8 μg DNA per 10 cm^2 dish using GeneJuice reagent (Novagen, Madison, WI), and proteins harvested 24 h after transfection. Cells were lysed using 200 μl of ProFound Lysis Buffer (Pierce, Rockford, IL) containing 1 mM Na_3VO_4, 1 mM PMSF, and 1× Protease Inhibitor Cocktail (Amersham BioSciences Corp., Piscataway, NJ). Cell lysates were clarified using 50 μl of glutathione–Sepharose 4B beads and quantified using the Bradford method [20].

A bacterial expression vector for full-length, myc-tagged TSAd was made by subcloning the TSAd cDNA into the pGEX-4T1 vector to create GST-TSAd. An expression vector for the proline-rich C-terminal portion (nucleotides 740–1167; amino acids 247–389) of TSAd was PCR-amplified from pcDNA3.1/SH2D2A plasmid and directionally cloned into the pGEX-4T-1 to create GST-$TSAd_{740}$ ($TSAd_{740}$). Plasmids for GST-SH2 domain expression of TSAd (TSAd-SH2), Lck (Lck-SH2), and PI3K (PI3K-SH2) were obtained from Dr. Philip King (University of Michigan, Ann Arbor, MI; [14]). BL21(DE3)LysS bacteria were transformed with bacterial expression vectors and expression was induced using isopropyl-β-D-thiogalactoside (IPTG; 0.1 mM for 2 h). Bacterial cells were pelleted at 4000*g* for 15 min (at 4 °C), resuspended in 1 ml TNE (Tris–Cl NaCl EDTA) buffer (20 mM Tris–Cl, pH 8.0, 1 mM EDTA, and 100 mM NaCl), and sonicated (4× 10 s; Sonic Dismembrator Model 100, Fisher Scientific). Protease Inhibitor Cocktail, Na_3VO_4 (to 1 mM), PMSF (to 0.1 mg/ml), and Triton X-100 (to 1% w/v) were added, and the suspension centrifuged at 14,000*g* for 15 min. Glutathione–Sepharose 4B beads (Amersham Biosciences; 50% suspension) were added to the supernatant and incubated for 2 h at 4 °C, centrifuged at 500*g* for 5 min at 4 °C, and washed three times with PBS. Proteins were quantified by running the samples on a protein gel that was then stained with Coomassie blue dye (0.25% Coomassie blue R250; 45% methanol; 10% acetic acid), and comparing against a BSA standard curve.

GST-protein bound to glutathione–Sepharose 4B beads was incubated with 1 mg of total cell protein from each condition overnight at 4 °C. The beads were washed four times with PBS (containing 1× Protease Inhibitor Cocktail), resuspended in modified 2× Laemmli loading buffer (125 mM Tris–Cl, pH 6.8, 4% w/v SDS, 22.5% v/v glycerol, 0.0015% w/v bromophenol blue, and 10% w/v β-mercaptoethanol) [21], incubated at 42 °C for 10 min, and loaded into a 12% SDS–polyacrylamide electrophoresis gel. Protein was transferred to nitrocellulose membranes and blocked for 1 h in 5% w/v non-fat milk in Tris–Cl-buffered saline with Tween 20 (TBS-T; 10 mM Tris–Cl, pH 8, 150 mM NaCl, and 0.1% Tween 20). Detection of protein by Western blot analysis was accomplished using primary mouse anti-human antibody against Flag (1:1000) or goat anti-GST-HRP (1:20,000; Amersham Biosciences, Piscataway, NJ). Sheep anti-mouse secondary antibody (POD-conjugated) was used for detection of Flag. The signal was detected by using ECLplus and a Storm phosphorimaging machine and software (Amersham Biosciences). All experiments used GST as a control to account for non-specific pull-down. Pull-down levels were normalized to total GST fusion-protein levels by staining with anti-GST antibody. Data are representative of at least three independent experiments.

Immunoprecipitation. COS-7 cells were washed with ice-cold phosphate-buffered saline and lysed in 1 ml of immunoprecipitation (IP) buffer [50 mM Hepes, pH 7.4, 100 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 1 mM Na pyrophosphate, 1 mM Na orthovanadate, 1× Protease Inhibitor Cocktail (Amersham), and 10 μg/ml PMSF (BD Biosciences Clontech)] for 30 min on ice, then clarified by centrifugation. Cell lysates (1 mg/ml) were first pre-cleared with protein A–Sepharose CL-4B (Amersham) followed by incubation with 4 μg anti-c-myc antibody (BD Biosciences Clontech) overnight at 4 °C. The immune complex was isolated with protein A–Sepharose CL-4B (2 h at 4 °C) and the beads washed three times with IP buffer. Proteins were separated by SDS–PAGE and analyzed by Western analysis. Protein was detected using polyclonal rabbit anti-human antibodies against Smad2 (1:1000; Zymed Laboratories Inc., South San Fransisco, CA), Smad3 (1:500; Zymed Laboratories Inc.), Smad4 (Upstate Biotechnology Inc., Charlottesville, VA), and mouse anti-c-myc (BD Biosciences Clontech).

Results and discussion

TSAd interacts with Smad2 and Smad3

To identify proteins that may be involved in the regulation of Smad signalling, we conducted a yeast two-hybrid screen of a human lymphocyte cDNA library using human Smad2 as bait. A positive clone, designated Y2H-P3, that interacted specifically with Smad2 encoded TSAd (data not shown). Sequencing and database analysis indicated that Y2H-P3 shared ~99% sequence identity with human *SH2D2A* (GenBank Accession No. NM_003975), which encodes TSAd. The Y2H-P3 cDNA lacked the first 16 nucleotides of the TSAd coding sequence, thus a cDNA clone IMAGE:4523238 (GenBank Accession No. BG389115) containing the human *SH2D2A* EST was obtained from Incyte Genomics (Wilmington, DE) and used to PCR amplify the full-length human *SH2D2A* coding sequence. PCR primers were designed to allow

directional subcloning of *SH2D2A* into the pcDNA3.1-myc-his vector (Invitrogen Life Technologies, Carlsbad, CA) to create pcDNA3.TSAd-myc (TSAd-myc).

To confirm the association of TSAd with Smad2 and to investigate the possible interaction of TSAd with other Smads, GST pull-down and co-IP assays were performed. COS-7 cells transfected with FLAG-tagged Smad2, Smad3 or Smad4 were used as the source of Smad protein for the GST pull-down assays. GST-TSAd interacted with Smad2 and Smad3 but not with Smad4, while no Smads were pulled down using GST alone (Fig. 1A). To directly examine protein–protein interactions in mammalian cells, COS-7 cells were transfected with FLAG-tagged-Smad2, -Smad3 or -Smad4 alone or together with TSAd-myc. Cell lysates were subjected to immunoprecipitation with anti-myc antibody, followed by Western blot analysis using anti-FLAG antibody. We determined that both Smad2 and Smad3 co-immunoprecipitate with TSAd (Fig. 1B). Similar to the GST pull-down experiments, no interaction between TSAd and Smad4 was detected in any of the co-IP experiments. Specific interaction of TSAd with full-length Smad2 and Smad3, but not Smad4, was detected in our systems where both TSAd and Smad molecules were overexpressed. These interactions suggest that TSAd may participate in Smad-mediated signalling; however, interaction between endogenous proteins in lung epithelium or T cells remains to be determined.

The TSAd-SH2 domain interacts with the N- and C-terminal domains of Smad2 and Smad3

Smad proteins have the ability to interact with numerous interacting or adapter proteins. These associations involve several different domains within the Smad proteins or their interacting partners. For example, the Smad MH1 domain can be bound by c-Jun, PY motifs within linker region interact with Smurf2 (WW domain), and the MH2 domain provides an interface with Smad anchor for receptor activation (SARA; Smad-binding domain; [6]), Disabled-2 (Dab2; PTB domain; [4]), and p300/CBP [22]. To further elucidate putative domains of interaction between the TSAd and Smad proteins, an expression construct

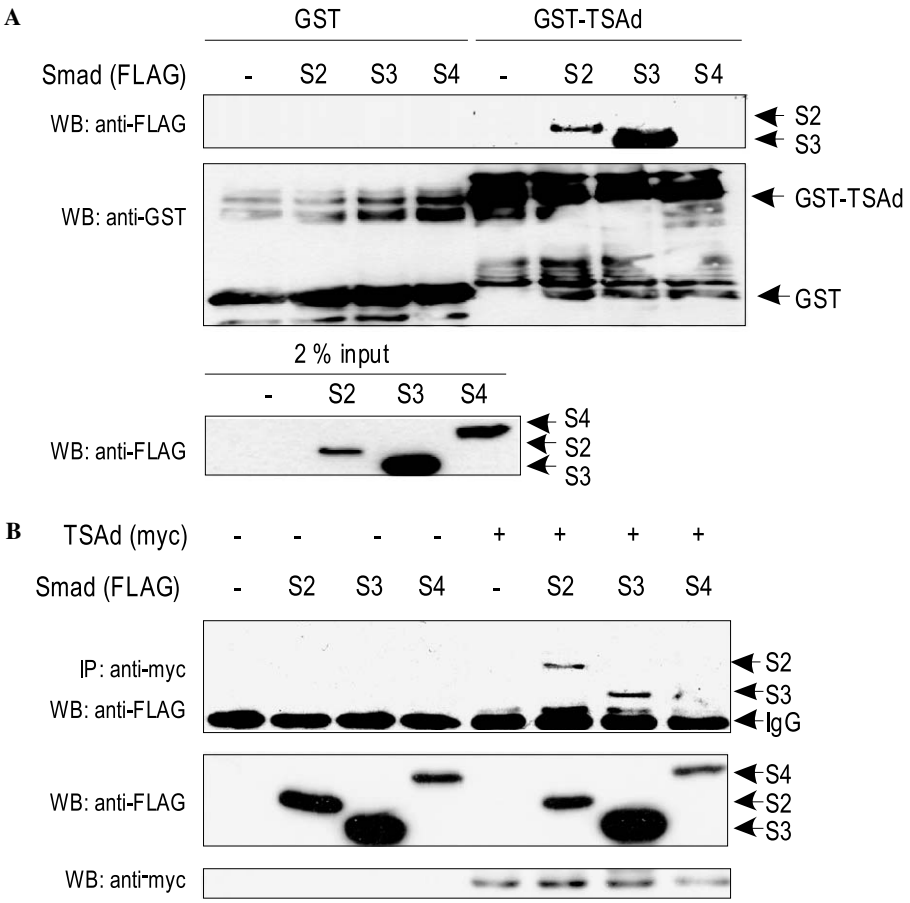

Fig. 1. TSAd interacts with Smad2 and Smad3. (A) GST-TSAd or GST was incubated with extracts from COS-7 cells untransfected (-) or transfected with FLAG-tagged Smad2 (S2), Smad3 (S3) or Smad4 (S4). Proteins associated with GST-TSAd and GST after pull-down with glutathione–Sepharose 4B were subjected to Western blot (WB) using anti-FLAG and anti-GST antibodies. WB of GST proteins or FLAG-tagged Smads from 2% of the COS-7 extracts (2% input) are shown. (B) S2 and S3 co-immunoprecipitated with TSAd-myc. COS-7 cells were transfected with S2, S3 or S4 alone or in combination with TSAd-myc. After 36 h whole cell lysates were subject to immunoprecipitation (IP) with anti-myc antibodies and Smad co-immunoprecipitation analyzed by WB using anti-FLAG antibodies. WB of FLAG-tagged Smads and TSAd-myc from 2% input is shown in the lower panels. IgG, immunoglobulin used for IP and detected by Western analysis.

encompassing the N-terminus of TSAd including the SH2 domain (TSAd-SH2) was initially used to assess interaction with full-length, N- and C-terminal Smad constructs. The N-terminal portion of Smad2 and Smad3 comprised the MH1 domain and most of the linker region, whereas the C-terminal portion of Smad2 and Smad3 contained the MH2 domain and a non-overlapping region of the linker domain. TSAd-SH2 pulled down full-length Smad2/3, N-Smad2/3, and C-Smad2/3 (indicated by arrowheads) (Fig. 2). Similar levels of GST-tagged proteins were detected by Western analysis as a loading control (Fig. 2C). In most experiments, the GST control pulled down background levels of Smad proteins. All experiments used GST as a control to account for non-specific pull-down.

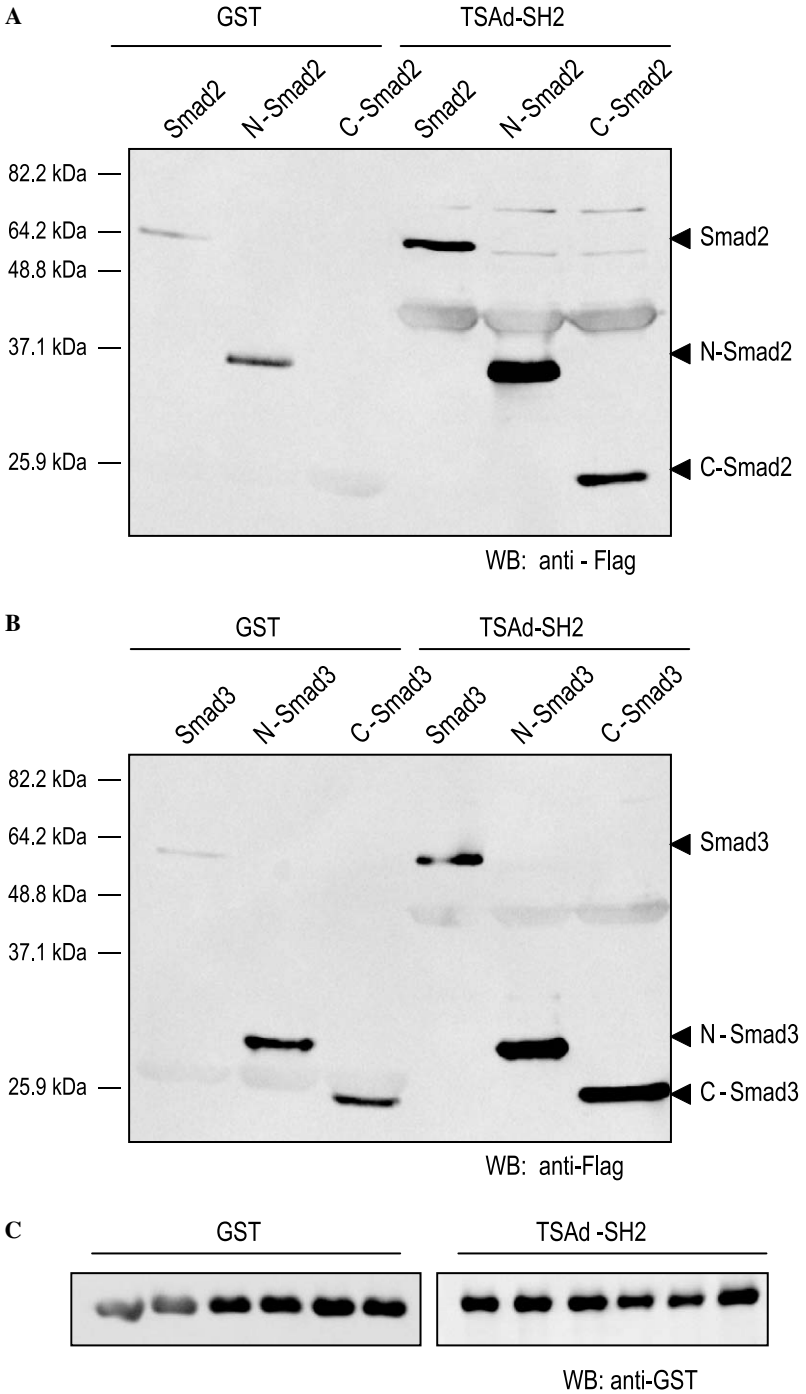

Fig. 2. The TSAd-SH2 domain interacts with the N- and C-terminal domains of Smad2 and Smad3. (A,B) Full-length, N- and C-terminal Smad2/3 constructs were used for TSAd-SH2 pull-down assays. Flag-tagged Smad proteins were detected with anti-Flag antibody by Western analysis. Background levels of Smad proteins were observed with GST alone. (C) Loading control from 10% of pull-down showed equal loading of GST proteins (GST or TSAd-SH2). GST fusion proteins were detected with anti-GST antibody by Western analysis.

These results suggest that TSAd-SH2 alone is sufficient to interact with the MH1 and MH2 domains of Smad proteins.

Smad2 or Smad3 differentially interact with type I and type III SH2 domains

The TSAd-SH2 domain is a type I SH2 domain [14]. Lad (murine TSAd) was originally cloned after a yeast two-hybrid screen to find Lck-interacting proteins [9]. Lck and PI3K SH2 domains were used to control for the specificity of the interaction between the Smad proteins and TSAd-SH2. The Lck-SH2 construct is also a type I SH2 domain, while the PI3K-SH2 construct is a type III SH2 domain [14]. Full-length, N- and C-terminal Smad2 and Smad3 constructs were used for pull-down assays with different GST-tagged proteins; GST protein alone (GST), TSAd-SH2, Lck-SH2, and PI3K-SH2. Equal loading was observed for the GST-domain proteins (Fig. 3A). As observed previously (Fig. 2), TSAd-SH2 pulled down higher levels of full-length Smad2 than the GST control (Fig. 3B). Minimal, but repeatable, interaction was observed between Lck-SH2 with full-length Smad2, whereas the level of interaction with PI3K-SH2 was comparable to GST alone. The TSAd-SH2, Lck-SH2, and PI3K-SH2 domains pulled down higher levels of N-Smad2 than GST control (Fig. 3B). When the C-Smad2 domain was used, TSAd-SH2 pulled down higher levels of C-Smad2 than GST control, while Lck-SH2 pulled down extremely low, but repeatable, levels of C-Smad2. PI3K-SH2 interaction with C-Smad2 was comparable to GST alone. These data show that Smad2 preferentially interacts with TSAd-SH2, compared to the other SH2 domain-containing proteins. This preferential interaction may be due to the additional 94 amino acid residues N-terminal to the TSAd-SH2 domain. While there was limited interaction between Lck-SH2 or PI3K-SH2 and full-length Smad2 or C-Smad2, greater interaction was observed with N-Smad2. These data imply that the N-terminus of Smad2 may provide a motif for interaction with SH2 domain-containing proteins, which may be masked by the full-length Smad2 protein.

TSAd-SH2 pulled down higher levels of full-length Smad3 than the GST control (Fig. 3C) as observed previously (Fig. 2). In addition, full-length Smad3 was also pulled down with Lck-SH2 and minimal, but repeatable, interaction was detected with the PI3K-SH2 domains (Fig. 3C). Similar results were observed with the N-Smad3 and C-Smad3 domains, with the exception that PI3K-SH2 interaction was no longer observed. It should be noted that lower levels of interaction between N-Smad3 and TSAd-SH2 were observed in the experiment shown (see Fig. 2 for typical result). Surprisingly, Lck-SH2 pulled down N-Smad3 and C-Smad3 in comparable levels to TSAd-SH2. These data suggest that Smad3 is capable of interacting with type I SH2 domain containing proteins, but has little affinity for type III SH2 domains. These results further imply that this interaction is unlikely due to the additional 94 amino acid residues N-terminal to the TSAd-SH2 domain, which are not present in Lck-SH2.

Smad2 or Smad3 differentially interacts with TSAd domains

Experiments to further elucidate the interaction between TSAd and Smad proteins utilized GST-TSAd, TSAd-SH2, and a C-terminal portion of TSAd ($TSAd_{740}$; C-terminal to the SH2 domain). Equal loading was observed for the GST-domain proteins (Fig. 3A). Full-length GST-TSAd was consistently detectable (arrowhead), although a high level of protein degradation was typically observed. As previously observed, GST-TSAd and TSAd-SH2 pulled down higher levels of full-length Smad2 and Smad3 than the GST control (Fig. 3B and C). Minimal interaction was observed between $TSAd_{740}$ and full-length Smad2 or Smad3. Whereas TSAd-SH2 interacts with N-Smad2 (Figs. 2A and 3B), minimal interaction was detected between N-Smad2 and GST-TSAd or $TSAd_{740}$ proteins. These data raise the possibility that the C-terminus of TSAd ($TSAd_{740}$) does not contain a motif supporting interaction with N-Smad2, and that full-length GST-TSAd may provide tertiary structure that masks the putative binding motif for N-Smad2. By contrast, GST-TSAd interacts with N-Smad3 (Fig. 3C), but minimal interaction was detected between N-Smad3 and $TSAd_{740}$. Therefore, $TSAd_{740}$ is unlikely to contain a motif supporting high affinity interaction with the N-terminal portions of Smad2 and Smad3. The fact that GST-TSAd is able to interact with N-Smad3, but not N-Smad2, highlights the possibility that GST-TSAd can associate with Smad2 and Smad3 through different motifs. When the C-Smad2 or C-Smad3 domain was used, TSAd-SH2, $TSAd_{740}$, and GST-TSAd pulled down higher levels of C-Smad2 or C-Smad3 than GST control, suggesting that Smad2 and Smad3 share similar structural features that facilitate interaction with the different TSAd domains.

Overall, these results suggest a model for TSAd–Smad interaction that is facilitated by multiple domains. While the TSAd-SH2 protein consistently pulled down all Smad2 and Smad3 proteins, the $TSAd_{740}$ protein consistently pulled down the C-terminal portion, but not the full-length or N-terminal portion, of Smad2 and Smad3. The interaction between $TSAd_{740}$ and the MH2 domain of Smad2 or Smad3 may be facilitated by the hydrophobic corridor of the MH2 domain, which interacts with proline-rich sequences on other Smad-interacting factors, such as SARA or FAST2 [23]. This hydrophobic corridor may interact with the proline-rich domain in the C-terminus of TSAd, which is contained within the full-length TSAd and $TSAd_{740}$ proteins. The reduced ability of $TSAd_{740}$ to interact with the full-length Smad proteins may be due to Smad protein folding resulting in masking of the interaction motif. Additionally, our data indicate that the TSAd-SH2 protein contains structural features that support interaction with the both MH1 and MH2 domains, or alternatively with non-overlapping portions of the Smad

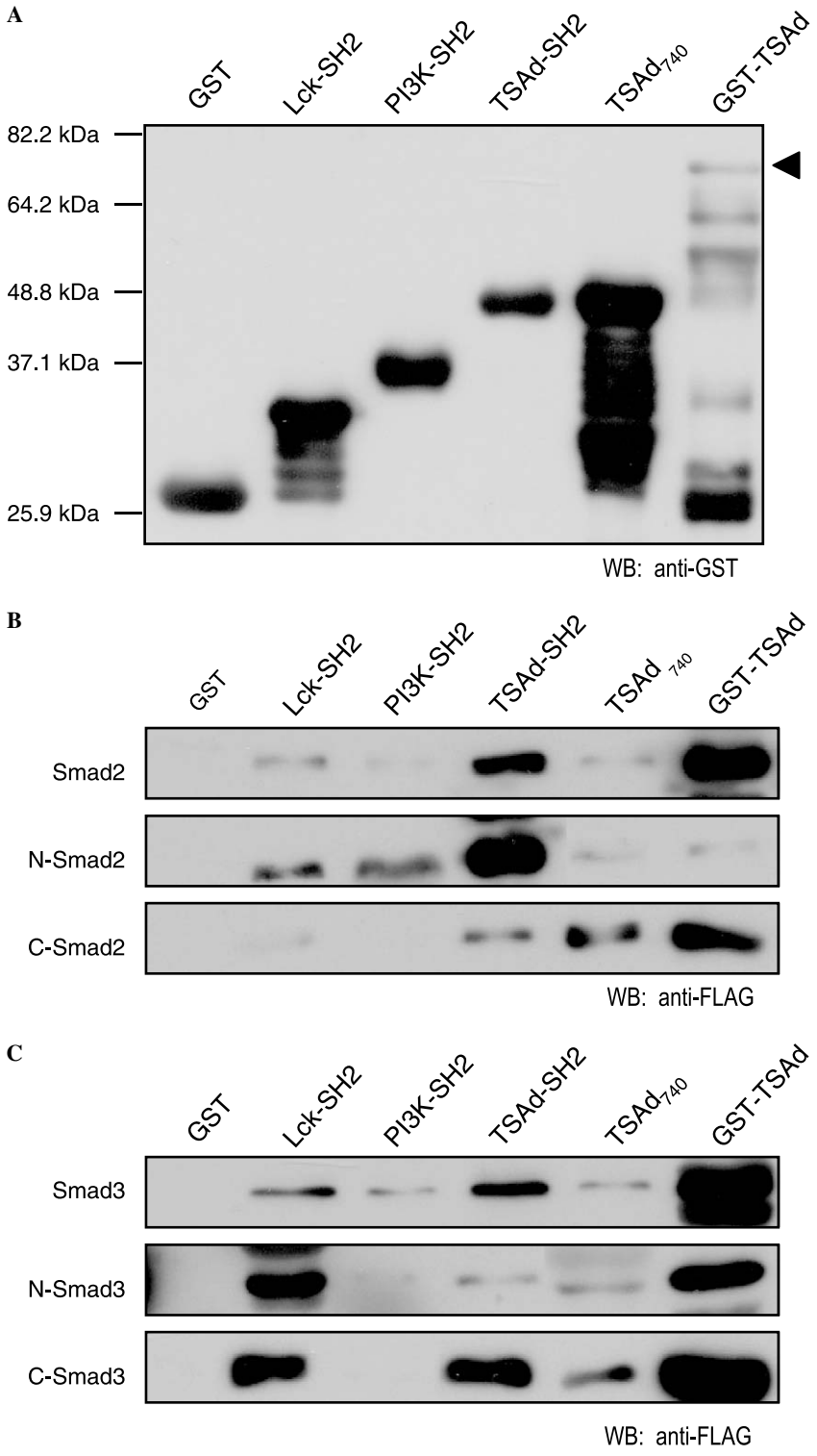

Fig. 3. Assessing Smad2 or Smad3 interaction with SH2 subtypes and TSAd domains. (A) GST fusion proteins were detected with anti-GST antibody by Western analysis: GST protein alone (GST), Lck-SH2, PI3K-SH2, TSAd-SH2, $TSAd_{740}$, GST-TSAd (arrowhead). (B,C) Full-length, N- and C-terminal Smad2 and Smad3 constructs, respectively, were used for GST pull-down assays. Flag-tagged Smad2/3 proteins were detected with anti-Flag antibody by Western analysis.

linker region. SH2 domains interact with phosphorylated tyrosine residues on other proteins. Potential phosphorylation of the Smad proteins may exist due to basal signalling activity stimulated by growth factors in serum. The interaction between TSAd-SH2 and the Smad proteins may therefore be a result of post-translational tyrosine phosphorylation of the Smad proteins in cells grown in the presence of serum. Under basal conditions, Smad

proteins may be phosphorylated within the linker region [24–26]. This may explain the ability of the TSAd-SH2 domain to pull-down both N- and C-terminal portions of Smad2 and Smad3 because they contain part of the linker and additional phosphorylated tyrosine sites. Therefore, the Smads being pulled down by the TSAd constructs may be an enriched fraction of Smads that are phosphorylated. Our results also indicate that Lck interacts with Smad2 and Smad3. These experiments may have uncovered a novel interaction between Lck and the Smad proteins, or these interactions may be due to the fact that the type I SH2 domains of TSAd and Lck are merely conserved sufficiently to facilitate interaction with Smad proteins. By contrast, the PI3K type III SH2 domain did not support a high level of interaction with the Smad proteins. Experiments to investigate interaction between the endogenous proteins are required to provide greater information about the novel interaction between SH2-containing proteins and Smad proteins. Furthermore, additional research must be done to elucidate the functional impact of TSAd on Smad-dependent signalling activity.

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