Conserved role for 14-3-3ε downstream of type I TGFβ receptors

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Abstract ***Schistosoma mansoni*** **receptor kinase-1 (SmRK1) is a divergent type I transforming growth factor β (TGFβ) receptor on the surface of adult parasites. Using the intracellular domain of SmRK1 as bait in a yeast two-hybrid screen we identified an interaction with *S. mansoni* 14-3-3ε. The interaction which is phosphorylation-dependent is not specific to schistosomes since 14-3-3ε also binds to TβRI, the human type I TGFβ receptor. 14-3-3ε enhances TGFβ-mediated signaling by TβRI and is the first TβRI-interacting non-Smad protein identified that positively regulates this receptor. The interaction of 14-3-3ε with schistosome and human TβRI suggests a conserved, but previously unappreciated, role for this protein in TGFβ signaling pathways.**

Key words: 14-3-3ε; Transforming growth factor β receptor; Scaffold protein; *Schistosoma mansoni* receptor kinase-1; *Schistosoma mansoni*

1. Introduction

Schistosoma mansoni receptor kinase-1 (SmRK1) is a divergent member of the receptor serine threonine kinase (RSTK) family [1]. Mammalian members of the RSTK family are receptors for the transforming growth factor β (TGFβ) family of ligands, which includes TGFβ's, activins, bone morphogenetic proteins (BMPs) and other factors that play prominent roles in governing growth and development. TGFβ receptors are classed as type I or type II (TβRI or TβRII) based on functional and structural characteristics [2]. Both TβRI and TβRII have a short cysteine-rich extracellular domain, a single transmembrane domain and an intracellular serine/threonine kinase domain. TβRIs are characterized by a highly conserved GS domain; a repetitive glycine–serine motif which is phosphorylated on activation. TβRIIs have a serine/threonine-rich C-terminus, are constitutively active and are responsible for ligand binding [2]. SmRK1 has previously been characterized as TβRI [1]. *S. mansoni*, a parasitic trematode, is the earliest diverging organism in which a member of the TGFβ receptor family has been identified.

Transduction of signal by RSTKs requires the ligand-mediated formation of a heteromeric complex between receptors I and II. TGFβ ligands can give rise to multiple outcomes in cells but the precise mechanisms by which all of the pleiotropic effects are mediated remain incompletely understood. In the most well characterized signaling pathway, TβRI, activated through phosphorylation by TβRII, phosphorylates Smad proteins which mediate signal transduction by translocation to the nucleus and alteration of gene expression to elicit the specific ligand response (reviewed in [3]). However, it is becoming increasingly clear that other pathways can be engaged by RSTKs. For example, both TGFβ and BMP can initiate activation of TGFβ-activated kinase, a MAPKKK (mitogen-activated protein kinase kinase kinase), with resultant activation of JNK and p38 kinases [4,5]. Members of the Ras and Rho families of small GTP-binding proteins have also been implicated in TGFβ signaling and certain MAPKs, such as ERK1 and SAPK-JNK, are activated by TGFβ in certain cell types [6,7]. These observations demonstrate the existence of cross talk between TGFβ receptors and multiple signaling pathways. However, in most cases the molecular mechanisms which link the TGFβ receptors and elements of these signaling cascades remain poorly defined.

As little is known about TGFβ signaling in *S. mansoni*, we used the yeast two-hybrid assay to identify proteins that interact with the intracellular domain of SmRK1. One of the clones identified encoded *S. mansoni* 14-3-3ε, which was shown to bind SmRK1 in vitro. This interaction is sufficiently conserved to cross species, since *S. mansoni* 14-3-3ε binds human TβRI both in vitro and in vivo. The functional consequence of this interaction is enhanced TGFβ-mediated signal transduction by TβRI. With the exception of Smads, 14-3-3ε is the first TβRI-interacting protein shown to activate signaling by this receptor.

2. Materials and methods

2.1. Two-hybrid assay in yeast

An adult *S. mansoni* cDNA library was screened with an intracellular domain of SmRK1 (amino acids (aa) 168–594), fused in-frame to the 3′ end of the DNA-binding domain of LexA. *Saccharomyces cerevisiae* EGY48 was first transformed with the reporter plasmid p8opLacZ, then subsequently co-transformed with the bait plasmid pLexA–SmRK1 and the cDNA library in pB42AD expression vector (Clontech). Positive co-transformants were selected on synthetic dropout (SD galactose/raffinose–Leu–His–Trp–Ura) plates, and confirmed by detection of β-galactosidase activity on plates with X-GAL. Candidate plasmids purified from yeast were sequenced by the dideoxy method.

2.2. Isolation of 14-3-3ε and sequence analysis

One positive clone encoded a fragment of DNA (115 bp) with homology to 14-3-3 proteins. The 14-3-3 sequence was completed by polymerase chain reaction (PCR) amplification of *S. mansoni* cDNA

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Abbreviations: SmRK1, *Schistosoma mansoni* receptor kinase-1; RSTK, receptor serine threonine kinase; TGFβ, transforming growth factor β; BMP, bone morphogenetic protein; TβRI, type I TGFβ receptor; TβRII, type II TGFβ receptor; GST, glutathione *S*-transferase; AP, alkaline phosphatase

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library in pB42AD, using a 5′ pB42AD primer and a 3′ 14-3-3-specific primer. The resulting PCR product was sub-cloned into PCRII (Invitrogen) and subjected to DNA sequencing. Full length 14-3-3ε was PCR amplified from *S. mansoni* first strand cDNA, sub-cloned into PCRII and re-sequenced for accuracy. PCR amplification of mouse cDNA using 14-3-3ε-specific primers yielded no bands, demonstrating that the 14-3-3ε identified was of schistosome origin and not a mouse gene contaminant of the *S. mansoni* cDNA library. *S. mansoni* 14-3-3ε was assigned GenBank accession number AF195529.

2.3. Expression plasmids

14-3-3ε was cloned into the *Bam*HI and *Xho*I sites of pGEX-4T-l (Amersham Pharmacia Biotech). The N-terminal FLAG-tagged 14-3-3ε (pCMV2-14-3-3ε-FLAG) was generated by sub-cloning at *Not*I and *Bam*HI sites into pCMV2 FLAG (Sigma). HA-tagged TβRI (pCMV5-TβRI-HA) and His-tagged TβRII (pCMV5-TβRII-His), provided by Dr. J. Massague, were used for PCR amplification of receptor intracellular domains. The intracellular domains of SmRK1 (aa 168–594) TβRI (aa 151–503) and TβRII (aa 195–567) were PCR amplified with primers placing an ATG within a Kozak consensus sequence at the 5′ end, and sub-cloned into PCRII plasmid.

2.4. In vitro interaction studies

Glutathione *S*-transferase (GST) and GST–14-3-3ε fusion proteins were produced from pGEX-4T-l-transformed bacteria, and bound to glutathione–Sepharose 4B beads as described [8]. The PCRII plasmids encoding the receptors were transcribed from the T7 promoter, translated and ^{35}S-labeled using the TNT rabbit reticulocyte lysate kit (Promega). ^{35}S-Labeled receptors were added to glutathione–Sepharose bound to either GST or GST–14-3-3ε in a total volume of 500 μl TNEN buffer (20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP40) and incubated for 2 h at 4°C. Following adsorption the beads were washed and subjected to SDS–PAGE followed by fluorography to detect specifically bound ^{35}S-labeled receptor protein. ^{35}S-Labeled SmRK1 was de-phosphorylated by incubating with 50 U alkaline phosphatase (AP; Gibco) at 30°C for either 15 or 60 min.

2.5. Co-immunoprecipitation

COS-7 cells were plated at 2×10^5 cells/well of six-well plates 18–24 h prior to transient transfection with lipofectamine (Gibco) and either pCMV2-14-3-3ε-FLAG (1 μg DNA/well) alone, or in combination with PCMV5-TβRI-HA or PCMV5-TβRII-His (1 μg total DNA/well). 48 h post-transfection, cells were harvested in lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100) containing 50 mM sodium fluoride and 1 mM sodium orthovanadate plus protease inhibitors. Following centrifugation, lysates were incubated with anti-HA or anti-FLAG antibody at 4°C overnight, followed by protein A Sepharose beads for 4 h. Following adsorption, beads were washed with lysis buffer and subjected to SDS–PAGE followed by transfer onto Immobilon P membrane (Millipore). Co-immunoprecipitated proteins were detected by immunoblot with either anti-FLAG or anti-TβRII (Santa Cruz Biotechnology), followed by horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence.

2.6. Functional assays

The R1B cell line, provided by Dr. J. Massague, is a mutant derivative of mink lung epithelial cells which lacks TβRI [9]. R1B cells, maintained in modified Eagle's medium, containing 10% fetal bovine serum, were plated at 1.5×10^5 cells/well of six-well dishes 18–24 h prior to transfection. Cells were transfected using 0.3 μg of expression construct, 0.3 μg reporter DNA, 0.1 μg β-galactosidase construct for normalization of transfection efficiency and 3 μl FuGENE 6 (Roche) per well. Following overnight recovery, cells were serum-starved for 4 h, then incubated for 20 h in the presence of 500 pM TGFβ (Genzyme). At 48 h post-transfection, the cells were harvested in reporter lysis buffer (100 mM KPO_4 and 100 mM dithiothreitol). The luciferase activity of lysates was measured in a Berthold luminometer (Lumat LB9501); β-galactosidase activity in the same lysates was analyzed using GalactoLight Plus (Tropix) and values were derived from triplicate determinations.

3. Results

3.1. Identification of 14-3-3ε through its interaction with SmRK1 in the yeast two-hybrid assay and cloning of full length sequence

To identify proteins that interact with SmRK1, an adult *S. mansoni* cDNA library was screened in a yeast two-hybrid assay using the intracellular domain of SmRK1 as bait. Of the

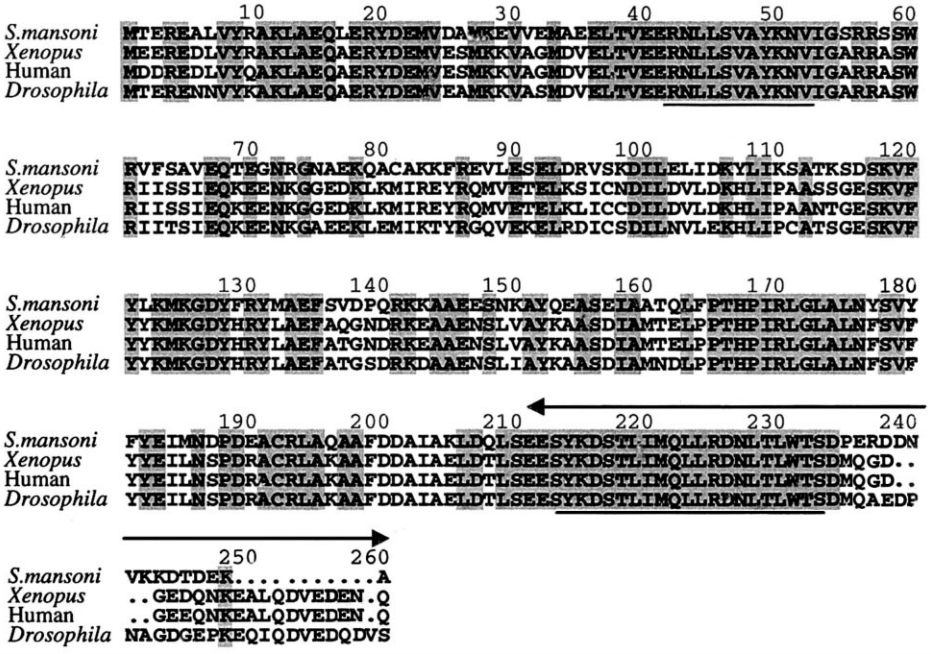

Fig. 1. Alignment of predicted amino acid sequence of *S. mansoni* 14-3-3ε with mammalian and *Drosophila* 14-3-3εs. Sequences were aligned using the Clustal function of the MegAlign program (DNASTAR Inc.). GenBank™ accession numbers for the 14-3-3ε sequences are *S. mansoni*, AF195529; *Xenopus*, AAC41251; human, U43399; and *Drosophila*, P92177. Identical amino acids shared by all four proteins are shaded. Two motifs characteristic of 14-3-3 family proteins are underlined. A double-headed arrow indicates the C-terminal region of *S. mansoni* 14-3-3ε identified in the two-hybrid screen.

resulting 17 positive clones that interacted with SmRK1, one encoded a protein with homology to 14-3-3 proteins. The majority of the 16 remaining positive clones shared no identity with known proteins; clones encoding protein interaction domains have been selected for further study. The 14-3-3 clone yielded a 115 bp DNA fragment with homology to the C-terminus of 14-3-3s, and included an in-frame stop codon. The remaining 14-3-3 sequence was obtained by primer extension, using 14-3-3- and vector-specific primers in PCR amplification of the schistosome cDNA library. Full length *S. mansoni* 14-3-3 amplified from first strand cDNA is 750 bp in length and codes for a protein of 249 amino acids, with an apparent molecular weight of 29 kDa. A search of GenBank™ using BLAST and comparison of the schistosome 14-3-3 with other 14-3-3 sequences revealed maximum similarity with *Drosophila* and mammalian 14-3-3ε homologs (Fig. 1). The deduced amino acid sequence of schistosome 14-3-3 shares 65, 64 and 64% identity with *Drosophila*, *Xenopus* and human 14-3-3ε sequences, respectively. As our SmRK1-interacting 14-3-3 has most identity with 14-3-3ε family members, we termed it *S. mansoni* 14-3-3ε. The two signature motifs characteristic of the 14-3-3 family of proteins are shown underlined in Fig. 1; these sequences are identical in all four sequences.

3.2. 14-3-3ε and SmRK1 interact directly in vitro in a phosphorylation-dependent manner

To confirm the interaction detected in yeast we assessed the ability of SmRK1 to interact with 14-3-3ε in vitro. ^{35}S-Labeled SmRK1 interacted directly with 14-3-3ε fused to GST, but not to GST alone (Fig. 2, lane 1). 14-3-3 proteins bind their target proteins at conserved binding sites containing a phosphoserine or phosphothreonine residue that plays a key role in the interaction [10,11]. SmRK1 does not contain any previously described 14-3-3 binding site, therefore, we aimed to determine whether phosphorylation of SmRK1 was important for its interaction with 14-3-3ε. ^{35}S-Labeled SmRK1 was treated with AP, and the ability of this de-phosphorylated SmRK1 to interact with GST–14-3-3ε was assessed (Fig. 2, lanes 2 and 3). AP treatment of SmRK1 for 15 min resulted in

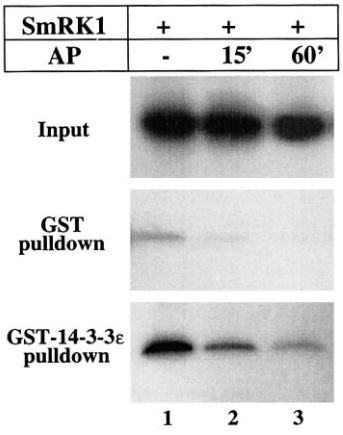

Fig. 2. Association of *S. mansoni* 14-3-3ε and SmRK1 is dependent on phosphorylation of SmRK1. ^{35}S-Labeled SmRK1 intracellular domain was untreated or treated with AP for 15 or 60 min and incubated with glutathione–Sepharose-coupled GST (middle panel) or GST–14-3-3ε (bottom panel). Following washing, eluted and input (top panel) proteins were analyzed by SDS–PAGE and fluorography. The top panel shows 20% of the input ^{35}S-labeled receptor used in each of the adsorption experiments.

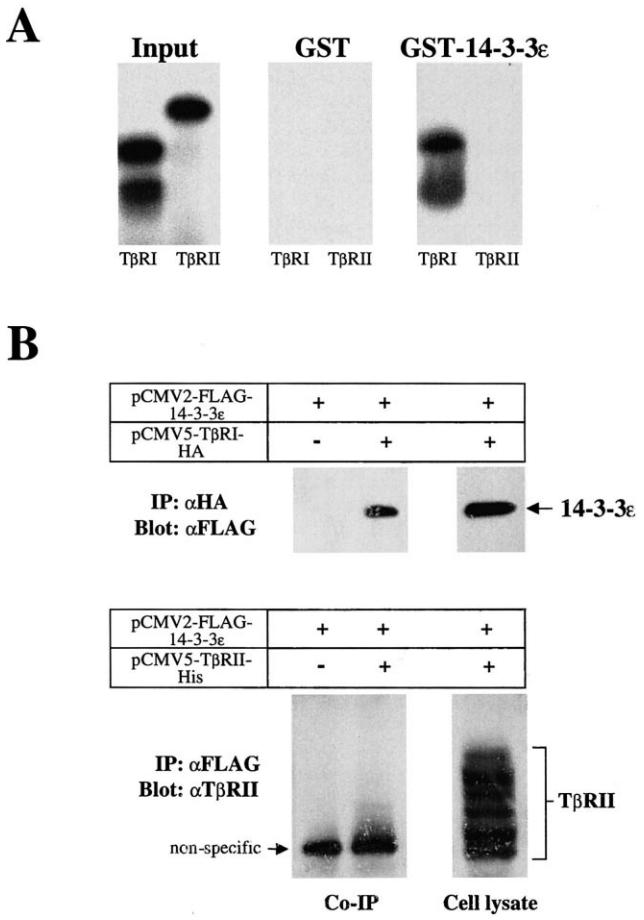

Fig. 3. Interaction of *S. mansoni* 14-3-3ε with TβRI and TβRII in vitro and in vivo. A: In vitro translated ^{35}S-labeled TβRI and TβRII intracellular domains were incubated with glutathione–Sepharose-coupled GST (middle panel) or GST–14-3-3ε (right panel); bound proteins were subject to SDS–PAGE and fluorography. B: COS-7 cells were transfected with pCMV2-14-3-3ε-FLAG alone, or in combination with pCMV5-TβRI-HA (top panel) or pCMV5-TβRII-His (bottom panel). Left panels show co-immunoprecipitations (Co-IP) and right panels show portions of clarified cell lysates to determine expression levels of 14-3-3ε and TβRII. Top: anti-HA immunoprecipitates (IP) analyzed for the co-precipitation of 14-3-3ε by immunoblotting with anti-FLAG antibody. Bottom: anti-FLAG immunoprecipitates analyzed for the presence of TβRII by immunoblotting with anti-TβRII antibody. The anti-TβRII antibody reacts with auto-phosphorylated TβRII resulting in a laddered binding pattern.

a greatly decreased level of SmRK1 association with GST–14-3-3ε as compared to untreated SmRK1, and when AP treatment was increased to 1 h, the association of SmRK1 and GST–14-3-3ε was almost completely abrogated (Fig. 2, lane 3). This correlation between SmRK1 de-phosphorylation and reduced binding to GST–14-3-3ε suggests that the interaction between SmRK1 and 14-3-3ε is, at least in part, phosphorylation-dependent.

3.3. 14-3-3ε interacts in vitro and in vivo with TβRI but not TβRII

Since schistosome 14-3-3ε has high homology with human 14-3-3ε, and the intracellular domain of SmRK1 shares 53% identity with this domain in TβRI, we sought to determine whether the 14-3-3ε–type I receptor interaction may be universal and species-independent. ^{35}S-Labeled TβRI interacted

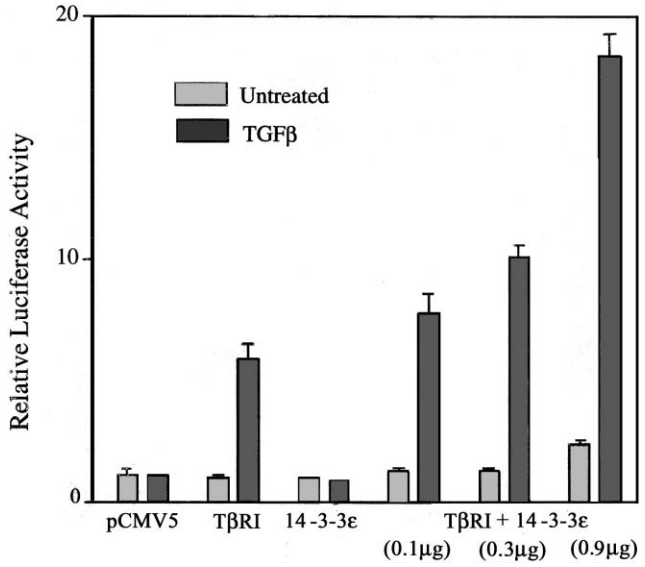

Fig. 4. *S. mansoni* 14-3-3ε enhances TGFβ-induced transcriptional activation of p3TP-Lux. R1B cells were transiently transfected with p3TP-Lux reporter, β-galactosidase construct and 0.3 μg of the expression constructs pCMV5-TβRI-HA or pCMV2-14-3-3ε-FLAG or with both constructs; pCMV5-TβRI-HA (0.3 μg) and increasing amounts of pCMV2-14-3-3ε-FLAG (0.1, 0.3 and 0.9 μg). Cells were subsequently incubated for 20 h in the presence of 500 pM TGFβ and relative luciferase activity was measured in cell lysates. Luciferase activity was normalized to β-galactosidase. Data are the mean from triplicate determinations of a representative experiment.

directly with *S. mansoni* 14-3-3ε fused to GST, but not to GST alone (Fig. 3A). In contrast, the TβRII did not interact with schistosome 14-3-3ε (Fig. 3A). To determine whether this in vitro interaction might also occur in vivo we performed co-immunoprecipitation studies. FLAG-tagged 14-3-3ε was expressed in COS-7 cells, alone or in combination with either HA-tagged TβRI or His-tagged TβRII. An anti-HA immunoprecipitation, which allowed purification of TβRI (data not shown), was followed by an anti-FLAG immunoblot for 14-3-3ε detection. 14-3-3ε was specifically co-precipitated by TβRI, confirming the interaction detected in vitro (Fig. 3B, top). In contrast, an anti-FLAG immunoprecipitation of 14-3-3ε followed by an anti-TβRII immunoblot showed no significant TβRII co-precipitation (Fig. 3B, bottom), demonstrating that 14-3-3ε interacts directly and specifically with TβRIs.

3.4. 14-3-3ε enhances TGFβ-mediated signaling by TβRI

To ascertain the functional relevance of the interaction between 14-3-3ε and TβRIs we performed studies with the TGFβ-responsive reporter plasmid p3TP-Lux, which contains elements from the PAI-1 promoter and drives expression of a luciferase reporter gene [12]. R1B cells lack TβRI and are unresponsive to TGFβ [9]. Transfection of the cells with TβRI restores responsiveness to TGFβ (Fig. 4). In the absence of TβRI, expression of *S. mansoni* 14-3-3ε alone had no effect on luciferase activity. However, when TβRI and 14-3-3ε were co-expressed, a TGFβ-induced increase in luciferase activity was observed. This increase in activity is significantly more than when the cells were transfected with TβRI alone and, moreover, activity is enhanced in a 14-3-3ε dose-dependent manner (Fig. 4). These results suggest that 14-3-3ε acts as a positive regulator of transcription activity in response to TGFβ.

4. Discussion

We report the identification of the ε isoform of 14-3-3 from *S. mansoni* and show that it interacts with the type I RSTK, SmRK1. Furthermore, 14-3-3ε interacts with and enhances TGFβ-mediated signaling by TβRI, as measured by activation of the p3TP-Lux promoter. The 14-3-3 family, which includes nine highly homologous members in mammals (α, β, γ, δ, ε, η, σ, τ and ζ), form dimers and are thought to function in a wide range of signaling pathways by acting as molecular scaffolds or chaperones [13]. 14-3-3ε has been reported as the isoform most likely to have retained the characteristics of the ancestral 14-3-3 gene [14].

S. mansoni 14-3-3ε, which shares 64% amino acid identity with human 14-3-3ε, binds to and enhances signaling by human TβRI. Conservation of 14-3-3 functionality across species has previously been demonstrated in yeast. *S. cerevisiae* has two genes encoding 14-3-3 proteins, *BMH1* and *BMH2*; and disruption of both genes is lethal [15]. A number of 14-3-3 isoforms from *Arabidopsis* complement this double disruption, as does human 14-3-3τ and a unique 14-3-3 protein from *Dictyostelium discoideum*, indicating the conserved nature of 14-3-3 function among different isoforms and different species [16].

A number of proteins that interact with mammalian type I receptor have been identified by yeast two-hybrid screens; none of these previously described interacting proteins were found in our screen of a schistosome cDNA library employing SmRK1 as bait. The immunophilin FKBP12 is the interacting protein most commonly detected, however, SmRK1 lacks the identified FKBP12 binding site, reflecting the divergent nature of this schistosome type I receptor [17]. Our identification of 14-3-3 as a type I receptor-interacting protein, yielded by a schistosome library screen, may reflect broad genomic differences between schistosomes and mammals. An interaction between TGFβ receptors and 14-3-3 proteins has not previously been reported, however results of a yeast complementation study have suggested a role for 14-3-3 in TGFβ signaling. *S. cerevisiae* strain DS9-22, defective in inositol-1-phosphate synthase and requiring *myo*-inositol for growth was complemented by clones expressing human 14-3-3, TβRII and the A subunit of protein phosphatase 2A; the Bα protein phosphatase 2A subunit interacts with TβRI [18,19]. These results suggest that all three proteins function in a common pathway.

TGFβ is a potent inhibitor of cell cycle progression in many cell types. 14-3-3 proteins also play a role in promoting cell cycle arrest through binding Cdc25. Cdc25 de-phosphorylates the cyclin-dependent kinase Cdc2, required for the initiation of mitosis. 14-3-3 binding results in the cytoplasmic sequestration of Cdc25 and its 'functional inactivation', thus maintaining cell cycle arrest [20]. PP2A also de-phosphorylates Cdc25, and its function is regulated by the Bα subunit, a protein which also interacts with TβRI [21,19]. Interaction of BαPP2A and 14-3-3 with TβRI, may provide a link between TGFβ receptor ligation and cell cycle arrest.

14-3-3 proteins have been shown to bind phophoserine residues with the consensus binding sites RSX*p*SXP and RXY/FX*p*SXP in many target proteins [10,11]. Recently, a number of non-consensus 14-3-3 binding sites have also been reported [10]. SmRK1 and TβRI contain neither a consensus motif nor

any other previously identified 14-3-3 binding sites. To determine whether phosphorylated residues are important in the SmRK1–14-3-3ε interaction, the ability of AP treated SmRK1 to interact with 14-3-3ε was ascertained. Concomitant with a time-dependent de-phosphorylation of SmRK1, a strong reduction in the amount of SmRK1 bound to 14-3-3ε was observed. However, AP treatment did not completely abolish binding, suggesting either the incomplete removal of phosphate or the presence of an additional phosphatase-insensitive 14-3-3ε binding site. SmRK1 contains at least one novel 14-3-3 binding site, which is likely to include either phosphoserine or phosphothreonine residues.

The 14-3-3ε clone identified by SmRK1 screening encoded only the C-terminal 38 amino acids of 14-3-3ε, indicating that this region is a binding site for SmRK1 and that the 14-3-3ε C-terminus is sufficient for SmRK1 binding. This is consistent with the finding that the 14-3-3 helix αI, which is retained within the C-terminal 38 amino acids, has been shown to be the region responsible for extensive interaction with phosphopeptide [22], and interaction studies with human 14-3-3τ have shown the C-terminal 15 amino acids to be sufficient for efficient binding of Cbl, Raf and phosphatidylinositol 3-kinase (PI3K) [23].

14-3-3 proteins have been found in the majority of important signaling pathways in cells, where they associate with a number of key signaling proteins and cell cycle regulators. These include Raf, PKC, MEKKs, PI3K, Bcr and Bcr-Abl, Cdc25, A20 and BAD, although the functional consequence of many 14-3-3–signaling protein interactions remains to be defined [13]. With the exception of Smads 2 and 3, which act as downstream substrates of TβRI to activate transcription activity, all previously identified TGFβ receptor-interacting proteins either have no effect on TGFβ-induced TβRI signaling (α subunit of farnesyl transferase), or act to inhibit transcription activity (FKBP12, BαPP2A, STRAP, TRAP and TRIP-1) [19,24–26]. Conversely 14-3-3ε acts to enhance TGFβ-induced signaling by TβRI.

In conclusion, conservation of 14-3-3's interaction with TβRIs from organisms as diverse as schistosomes and humans indicates that 14-3-3 is likely to play a role in TGFβ signal transduction in most, if not all, animal species. We aim to investigate the functional significance of the SmRK1–14-3-3 interaction in schistosomes.

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