Eukaryotic Initiation Factor 2α Subunit Associates with TGFβ Receptors and 14-3-3ϵ and Acts as a Modulator of the TGFβ Response[†,‡]

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ABSTRACT: *Schistosoma mansoni* receptor kinase 1 (SmRK1) is a divergent member of the TGFβ receptor family. Intracellular proteins that associate with these receptors are likely to play an important role in signaling. 14-3-3ϵ is a previously described cytoplasmic protein, which associates with both SmRK1 and the human type I TGFβ receptor (TβRI); overexpression of 14-3-3ϵ leads to enhanced TGFβ-mediated signaling by TβRI. We now describe the identification of *S. mansoni* eukaryotic translation initiation factor 2α subunit (eIF2α), through its interaction with SmRK1 in a yeast two-hybrid assay. *S. mansoni* eIF2α also interacts with human TGFβ receptors. Strongest association was demonstrated with kinase inactive receptors, particularly the type II TGFβ receptor (TβRII). Both TβRI and TβRII phosphorylate eIF2α in vitro, at sites other than the previously described eIF2α phosphorylation sites. EIF2α also modulates signaling by TGFβ receptors; however, in contrast to 14-3-3ϵ, eIF2α overexpression inhibits the TGFβ-driven response. These data suggest a novel function for eIF2α in the TGFβ signaling pathway. In addition, we have demonstrated an independent interaction between eIF2α and 14-3-3ϵ. Coexpression of 14-3-3ϵ with eIF2α leads to the abrogation of the inhibitory effect of eIF2α on TGFβ-mediated signaling. The interaction of these two regulatory proteins with each other and with the TGFβ receptors and their relative expression levels are likely to be important in fine-tuning the regulation of TGFβ signal transduction.

Members of the transforming growth factor β (TGFβ) superfamily of ligands are multifunctional proteins that regulate many diverse biological processes, and play predominant roles in governing growth and development (*1*). These proteins exert their biological effects through the receptor serine threonine kinase (RSTK) family of transmembrane receptors, whose members have been identified across a wide range of species, from nematodes to humans. RSTKs are classified as type I or type II receptors, based on structural and functional characteristics (*2*). *Schistosoma mansoni* receptor kinase 1 (SmRK1) is a divergent member of the RSTK family (*3*). A type I TGFβ receptor has also been reported from the parasitic nematode *Brugia pahangi*, and two type I receptors (DAF-1 and SMA-6) and one type II receptor (DAF-4) are present in the free living nematode *Caenorhabditis elegans* (*4*, *5*).

TGFβ signaling is initiated when ligand binding to type II receptor induces the formation of a heteromeric complex composed of receptors I and II. Type II receptor then phosphorylates type I receptor on serine residues within the highly conserved "GS" domain, resulting in activation of type I receptor. In the recently characterized downstream signaling pathway, active type I receptor phosphorylates Smad proteins, which mediate signal transduction through translocation to the nucleus and alteration of gene expression, to elicit the specific ligand response (reviewed in ref *6*). SmRK1 shares most homology with type I receptors (up to 58% identity in kinase domains) and has a conserved GS domain. It also contains a unique insert in the juxtamembrane region, and a long C-terminal extension, and thus, has previously been classified as a divergent type I receptor (*3*).

TGFβ ligands can give rise to multiple outcomes in cells, but the precise mechanisms by which these pleiotropic effects are mediated remain incompletely understood. Although Smad proteins have been identified as a direct downstream pathway from RSTK receptors, a variety of other signaling pathways are involved in TGFβ signal transduction; an example is TGFβ-activated kinase, a MAPKKK activated by both TGFβ and bone morphogenetic proteins and whose activation results in the activation of JNK and p38 kinase (*7*, *8*). Extensive cross talk between the Smad pathway and other signaling cascades including the JAK−STAT pathway (*9*), mitogen-activated protein kinase pathways (*10*, *11*), and the glucocorticoid receptor pathway (*12*) has also been demonstrated.

Proteins not yet shown to be directly involved in TGFβ signal transduction, but which may play a regulatory role in receptor function, have been identified through efforts to identify signaling pathways. These proteins include the Bα

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[1] Abbreviations: TGFβ, transforming growth factor β; RSTK, receptor serine threonine kinase; SmRK1, *Schistosoma mansoni* receptor kinase 1; BαPP2A, Bα subunit of protein phosphatase 2A; TβRI, type I TGFβ receptor; TβRII, type II TGFβ receptor; eIF2α, eukaryotic translation initiation factor 2α subunit; GST, glutathione *S*-transferase; AP, alkaline phosphatase; PAI-1, plasminogen activator inhibitor-1.

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subunit of protein phosphatase 2A (BαPP2a), TRIP-1, STRAP, all WD domain containing proteins, the α subunit of farnesyl transferase, TRAP1, and the immunophilin FKBP12 (*13*−*17*). Although functional roles for many of these proteins have not yet been determined, functions for TRAP1, as a Smad 4 chaperone, and for FKBP12, as a negative regulator of receptor endocytosis have recently been described (*18*, *19*).

SmRK1 is located on the surface of *S. mansoni*, a parasitic trematode, where it is likely to play a role in mediating host−parasite interactions that are essential for the parasite's survival and development within its mammalian host. Recently, we have identified elements of a Smad signaling pathway in *S. mansoni* (SmSmad1 and SmSmad2) and demonstrated that SmRK1 is capable of signaling via SmSmad2 in response to human TGFβ (*20*). To identify additional elements in the SmRK1 signaling cascade, we have used the intracellular domain of the receptor as bait in a yeast two-hybrid screen. We previously reported the identification of two SmRK1-interacting proteins: SIP and 14-3-3ϵ (*21*, *22*). SIP is a novel schistosome protein, which is thought to function as an adapter molecule that links SmRK1 to other signaling proteins (*21*). 14-3-3ϵ also interacts with human type I TGFβ receptor (TβRI) and positively regulates TGFβ-mediated signaling by TβRI (*22*). In this study, we report the identification of a third SmRK1-interacting protein, *S. mansoni* eukaryotic translation initiation factor 2α subunit (eIF2α). This association also appears to be conserved across species, since schistosome eIF2α interacted with human TGFβ receptors. The strongest in vivo interaction was observed with kinase-inactive type II TGFβ receptor (TβRII). EIF2α inhibits signaling in response to TGFβ and is phosphorylated by both TβRI and TβRII in vitro. We also demonstrate an association between eIF2α and 14-3-3ϵ, and show that coexpression of 14-3-3ϵ prevents eIF2α's inhibition of TGFβ-mediated signaling. Our results suggest a novel function for the translation initiation factor in the TGFβ signaling pathway.

MATERIALS AND METHODS

Yeast Two-Hybrid Assay. An adult *S. mansoni* cDNA library in pB42AD expression vector (Clontech) was screened with the intracellular domain (amino acids 168−594) of SmRK1, 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, and then subsequently cotransformed with the bait plasmid pLexA-SmRK1 and the pB42AD cDNA library. Positive cotransformants were selected on synthetic drop-out (SD galactose/raffinose −Leu −His −Trp −Ura) plates and confirmed by the detection of β-galactosidase activity on plates with X-GAL. Candidate plasmids purified from yeast were sequenced by the dideoxy method.

Isolation of eIF2α and Sequence Analysis. Two positive clones encoded DNA fragments with homology to eukaryotic initiation factor 2α subunit (eIF2α). The largest clone of 1052 bp in length, contained a complete open reading frame including a stop codon and a poly A tail. Full-length eIF2α was PCR amplified from first strand adult *S. mansoni* cDNA, subcloned into PCRII (Invitrogen) and resequenced for accuracy. *S. mansoni* eIF2α was assigned GenBank accession no. AF376135.

Expression Plasmids. EIF2α was cloned into the *Bam*H1 and *Not*1 sites of pGEX-4T-1 (Amersham Pharmacia Biotech). N-terminal FLAG-tagged and C-terminal MYC-tagged eIF2α were generated by subcloning at *Eco*R1 and *Bam*H1 sites into pCMV2-FLAG (Sigma), and at *Bam*H1 and *Not*1 sites into pcDNA3.1 (Invitrogen), respectively. 14-3-3 constructs were generated as previously described (*22*). HA-tagged human type I TGFβ receptor (TβRI) and human type II receptor (TβRII) in pCMV5, kindly 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), TβRII (aa 195−567), and full-length 14-3-3 and eIF2α were PCR amplified with primers placing an ATG within a Kozak consensus sequence at the 5′ end and subcloned into PCRII for transcription with T7 polymerase and translation in vitro. Herpes simplex virus-1 protein UL32 in pcDNA3.1 was kindly provided by Dr. A. Reynolds. To generate the truncated eIF2α proteins, ΔN, lacking amino acids 1−115, and ΔC, lacking amino acids 157−328, PCR primers were designed to enable amplification of the coding sequence as follows: ΔN forward primer: 5′-GGTTAACCAGATCTTGAGG-3′; reverse primer: 5′-GGAGACCAAAATGGACTAAAGTGA-3′; ΔC forward primer: 5′-ATGCCAATACAGTGCAGATTC-3′; reverse primer: 5′-CGACATTTTCAAGAAAGTTGTAA-3′. PCR products were subcloned into PCRII plasmid, and truncations were verified by sequencing prior to in vitro translation. Serine 48 and 51 point mutations were introduced into pCMV2-eIF2α using QuikChange site-directed mutagenesis (Stratagene) and confirmed by sequencing. Kinase inactive TβRI and TβRII constructs were generated by introducing a single-point mutation in the kinase domain: TβRI; K232R, TβRII; K277R, as previously described (*23*, *24*), and generation of mutant constructs was confirmed by sequencing.

In Vitro Interaction Studies. Glutathione S-transferase (GST), GST-eIF2α, and GST-14-3-3 fusion proteins were produced from pGEX-4T-1-transformed bacteria, and bound to glutathione-sepharose beads (Amersham Pharmacia Biotech) as described (*25*). The PCRII and pcDNA3.1 plasmids encoding the receptor intracellular domains, UL32, 14-3-3, eIF2α or the truncated mutants, eIF2αΔN and ΔC, were transcribed from the T7 promoter and translated and ^{35}S-labeled using the TNT rabbit reticulocyte lysate kit (Promega). ^{35}S-Labeled proteins were added to glutathione-sepharose bound to either GST or GST fusion protein in a total volume of 500 μL of 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 protein. ^{35}S-Labeled receptors or eIF2α were dephosphorylated by incubating with 50 U alkaline phosphatase (Gibco) at 30 °C for 60 min. The reaction was terminated by the addition of sodium fluoride and sodium orthovanadate to final concentrations of 50 mM and 200 μM, respectively.

Coimmunoprecipitation. COS-7 cells were maintained in DMEM containing 10% fetal bovine serum, 2 mM glutamine, 100 μM nonessential amino acids, and 100 U/mL penicillin−streptomycin solution at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were plated at 1×10^6 cells/100-mm plate, 18−24 h prior to transient transfection with

lipofectamine (Gibco) and a total of 5 μg of DNA per plate. Cells to be metabolically labeled were washed twice with PBS at 24 h post-transfection, and incubated in DMEM −Cys −Met with 50 μCi/mL TRAN^{35}S-label (ICN Biomedicals, Inc.) overnight. Forty-eight hours post-transfection, all cells were harvested in lysis buffer plus protease and phosphatase inhibitors (*26*). Following centrifugation and preclearing of lysates with protein A or G sepharose beads (Amersham Pharmacia Biotech), anti-HA (Boehringer Mannheim), anti-FLAG (M2; Sigma), or anti-MYC (Invitrogen) monoclonal antibodies or anti-TβRII (C-16; Santa Cruz Biotechnology) polyclonal antibody was added and incubated at 4 °C overnight. Immune complexes were incubated with protein A or G sepharose beads for 4 h, and following adsorption, beads were extensively washed with lysis buffer and subjected to SDS−PAGE. Electrophoresis was followed by fluorography for detection of precipitated proteins from metabolically labeled cells, or transfer onto Immobilin P membrane (Millipore) for immunoblot. Coimmunoprecipitated proteins were detected by immunoblot with either anti-TβRII or anti-FLAG, followed by horseradish peroxidase-conjugated secondary antibody and ECL (Amersham Pharmacia Biotech).

In Vitro Kinase Assay. EIF2α and either TβRI or TβRII were immunoprecipitated from COS-7 cells and bound to protein A sepharose beads. Expression of eIF2α and the receptors was assessed by immunoblot and equivalent amounts of protein were used in the kinase assay. Beads were washed three times with lysis buffer and twice with kinase buffer (5 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$) and then incubated in kinase reaction mix containing 20−30 mCi (γ-^{32}P)-ATP (Amersham Pharmacia Biotech) in kinase buffer with 1 μM ATP in a final volume of 30 μL. Following incubation at room temperature for 30 min, beads were washed three times with kinase buffer and twice with lysis buffer, and boiled in sample buffer. Samples were resolved by SDS−PAGE, and phosphorylated proteins were visualized by autoradiography.

Functional Assays. Mink lung epithelial cells (Mv1Lu; CCl-64, ATCC) were maintained in modified Eagle's medium, Earles, (MEM) containing 10% fetal bovine serum, 2 mM glutamine, 100 μM nonessential amino acids and 100 U/mL penicillin−streptomycin solution. Cells were plated at 1.5×10^5 cells/well of 6-well dishes 18−24 h prior to transfection. Cells were transfected using 0.3 μg of expression construct, 0.3 μg of reporter plasmid, 0.1 μg of β-gal construct for normalization of transfection efficiency, and 3 μL of FuGENE 6 (Roche) per well. Following overnight recovery, cells were serum starved for 4 h and then incubated for 20 h in the presence or absence of TGFβ (R & D). Forty-eight hours post-transfection, the cells were harvested in reporter lysis buffer (100 mM KPO_4 and 100 mM dithiothreitol). 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).

RESULTS

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. Two of the resulting 17 positive clones encoded a protein with homology to eukaryotic translation initiation factor 2α subunit (eIF2α). The largest clone of 1052 bp contained a complete open reading frame of 987 bp, beginning with an ATG codon four nucleotides downstream of the 5′ end, and a long 3′ untranslated region, terminating with a poly A tail of 18 residues. The open reading frame encodes a protein of 328 amino acids with a predicted molecular weight of 37 kDa. A search of GenBank using BLAST and comparison of the schistosome eIF2α with other eIF2α sequences using the alignment program MegAlign revealed high homology with both mammalian and nonmammalian eIF2α homologues (Figure 1); therefore, we termed our SmRK1-interacting protein *S. mansoni* eIF2α. The deduced amino acid sequence of schistosome eIF2α shares 55, 54, 52, and 50% identity with mammalian, zebrafish, *Artemia*, and *Drosophila* eIF2α protein sequences, respectively, and most of the identical regions lie within the N-terminal half of the protein (Figure 1).

To confirm the SmRK1−eIF2α interaction detected in yeast, we assessed the ability of SmRK1 to directly interact with eIF2α in vitro. The ^{35}S-labeled in vitro-translated intracellular domain of SmRK1 interacted directly with eIF2α fused to GST but not to GST alone (Figure 2A, first lane). Since *S. mansoni* eIF2α has high homology with human eIF2α, and the intracellular domain of SmRK1 shares 53 and 30% identity with this domain in human TGFβ type I receptor (TβRI), and type II receptor (TβRII), respectively, we sought to determine whether this interaction may be universal among TGFβ receptors, and species-independent. The ^{35}S-labeled intracellular domains of both TβRI and TβRII interacted directly with GST-eIF2α but not with GST alone (Figure 2A). In contrast UL32, a herpes simplex virus-1 protein, included as a control, was not bound by GST-eIF2α (Figure 2A right panel). Protein−protein interactions are often phosphorylation dependent; therefore, we aimed to determine whether phosphorylation within SmRK1 and the human TGFβ receptors was important for the eIF2α interaction. The ^{35}S-labeled intracellular domains of SmRK1, TβRI, and TβRII were treated with alkaline phosphatase (AP), and the ability of these dephosphorylated receptors to interact with GST-eIF2α was assessed (Figure 2B). AP treatment of the receptors resulted in similar or increased levels of GST−eIF2α association as compared to the untreated receptors (Figure 2B). This suggests that the association of TGFβ receptors and eIF2α does not require phosphorylation and may even be stronger in the absence of phosphorylation.

To determine whether this in vitro interaction might also occur in vivo, we performed coimmunoprecipitation studies from metabolically labeled mammalian cells. SmRK1 expression level in mammalian cells is low as compared to that obtained with the human TGFβ receptors; therefore, we focused the remainder of our study on the interaction of eIF2α with TβRI and TβRII. FLAG-tagged eIF2α, alone or in combination with the full-length receptors; either HA-tagged TβRI or His-tagged TβRII were expressed in COS-7 cells. Coimmunoprecipitation of eIF2α with the receptors was not observed using either anti-FLAG to immunoprecipitate eIF2α, or anti-HA or anti-TβRII in receptor immunoprecipitations (data not shown). Having previously observed that eIF2α−receptor binding in vitro appears to be enhanced in the absence of phosphorylation, we repeated

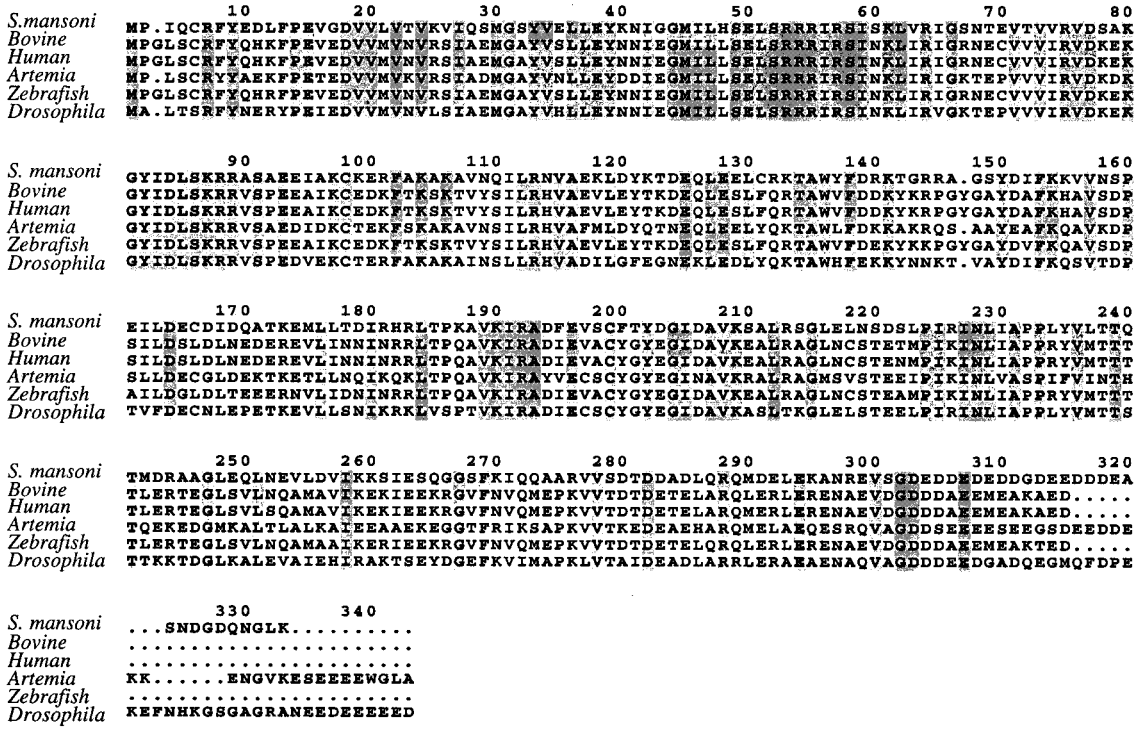

FIGURE 1: Alignment of predicted amino acid sequence of *S. mansoni* eIF2α with other eIF2α sequences. Sequences were aligned using the Clustal function of the MegAlign program (DNASTAR Inc.). GenBank accession numbers for the eIF2α sequences are *S. mansoni*: AF376135; bovine: P05199; human, NP_004085; *Artemia*: 227768; zebrafish: AF257517; *Drosophila*: P41374. Identical amino acids shared by all six proteins are shaded.

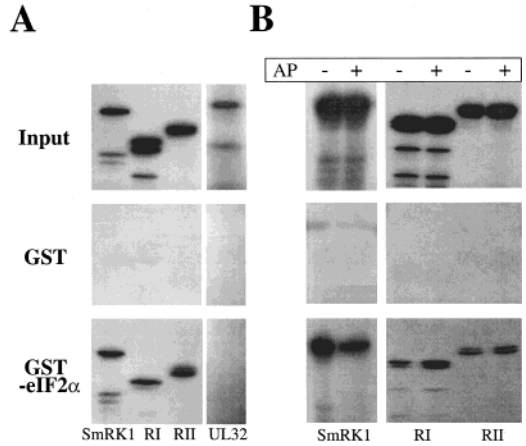

FIGURE 2: In vitro association of *S. mansoni* eIF2α with SmRK1, TβRI, and TβRII is not dependent on phosphorylation of the receptors. (A) eIF2α interacts with SmRK1, TβRI, and TβRII, but not UL32 in vitro. ^{35}S-labeled intracellular domains of SmRK1, TβRI (RI), and TβRII (RII), and full-length herpes simplex virus-1 protein UL32, as a negative control, were incubated with glutathione-sepharose-coupled GST (middle panel) or GST-eIF2α (bottom panel). Following extensive washing, bound 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 experiments. (B) Interaction of eIF2α with receptors is not phosphorylation dependent. ^{35}S-labeled receptors were untreated or treated with alkaline phosphatase (AP) for 60 min and incubated with glutathione-sepharose-coupled GST (middle panel) or GST-eIF2α (bottom panel). Following washing, eluted and input (top panel) proteins were analyzed by SDS−PAGE and fluorography. Results are representative of three individual experiments.

the coimmunoprecipitation studies using kinase-inactive mutants of the receptors (*23*, *24*) (Figure 3). No association with eIF2α was observed in an anti-HA immunoprecipitation of kinase inactive TβRI (TβRI-KR); whereas, an anti-FLAG immunoprecipitation of eIF2α revealed a faint TβRI-KR band (Figure 3A). These data suggest a weak association between eIF2α and kinase inactive TβRI. In contrast, both anti-TβRII and anti-FLAG immunoprecipitations demonstrated a strong association between kinase inactive TβRII (TβRII-KR) and eIF2α (Figure 3B). Coimmunoprecipitations were also assessed by immunoblot, and as before, very little association resulted between eIF2α and active TβRI or TβRII (data not shown). Immunoprecipitation of kinase inactive TβRI and TβRII, followed by immunoblot with anti-FLAG confirmed the weak eIF2α−TβRI-KR interaction and the much stronger association of eIF2α with TβRII-KR (Figure 3C).

The interaction between eIF2α and the receptors appeared to be stronger in the absence of kinase activity, suggesting that activation of TGFβ receptor signaling may result in eIF2α dissociation from the receptors. Phosphorylation may play a role in eIF2α dissociation; therefore, to determine whether eIF2α is a substrate for the kinase activity of TβRI and TβRII, we performed an in vitro kinase assay. Since association between eIF2α and kinase active receptors is very weak, double immunoprecipitations were performed prior to performing the kinase assay. EIF2α was phosphorylated in vitro by both TβRI and TβRII, but not in the absence of the receptors (Figure 4A,B). Immunoprecipitation of TβRII resulted in the coprecipitation of endogenous TβRI (Figure 4B). Autophosphorylation of both TβRI and TβRII was observed and appeared to be significantly enhanced in the presence of eIF2α (Figure 4A,B). When TβRII was expressed alone a faint band with similar migration to eIF2α was observed (Figure 4B, lane 2), suggesting that there may be an association between the two proteins. However, the interaction appears to be too transient in nature to be detected

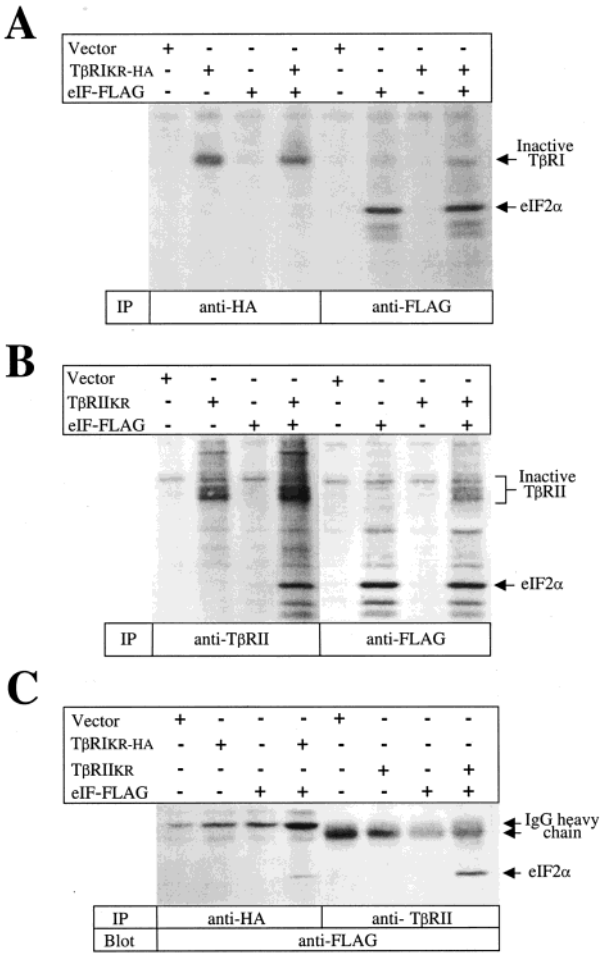

FIGURE 3: Interaction of eIF2α with kinase inactive TβRI and TβRII in mammalian cells. (A) Association of eIF2α with TβRI-KR. COS-7 cells were transfected with FLAG-tagged eIF2α or HA-tagged TβRI-KR either alone or together, or with empty vector as a control. Cells were metabolically labeled overnight and lysed, and the lysates were subjected to anti-HA or anti-FLAG immunoprecipitation (IP). No coprecipitation was observed with anti-HA IP. A faint TβRI-KR band was coprecipitated by anti-FLAG IP of eIF2α. (B) Association of eIF2α with TβRII-KR. COS-7 cells transfected with either FLAG-tagged eIF2α or TβRII-KR were metabolically labeled overnight and lysed. ^{35}S-labeled lysates were subjected to anti-TβRII or anti-FLAG IP. eIF2α was coprecipitated with TβRII-KR, and TβRII-KR was coprecipitated with eIF2α from the double transfections. (C) EIF2α association with TβRI-KR and TβRII-KR detected by immunoblot. COS-7 cells were transfected with vector alone, with eIF2α-FLAG alone, or in combination with TβRI-KR or TβRII-KR. Cells were lysed and immunoprecipitated by anti-HA or anti-TβRII to precipitate the inactive receptors. Coprecipitating eIF2α was detected by immunoblot (blot) with anti-FLAG antibodies. Immunoglobulin heavy chain is indicated.

by coimmunoprecipitation studies. Phosphorylation of eIF2α is one of the most important mechanisms for repressing global rates of protein synthesis. EIF2α serine 51 is the phosphorylation site responsible for the translation block (*27*). In addition, serine 48 appears important for the high affinity interaction between eIF2α and eIF2B (*28*). To determine whether serine 51 or serine 48 were phosphorylated by TβRI or TβRII, we generated the eIF2α mutants: S51A, S48A, and the double mutant, S48A,S51A. All three mutants acted as substrates for both TβRI and TβRII (Figure 4A,B), demonstrating that phosphorylation of eIF2α by the TGFβ receptors occurs at sites other than, or in addition to, serine 48 and 51.

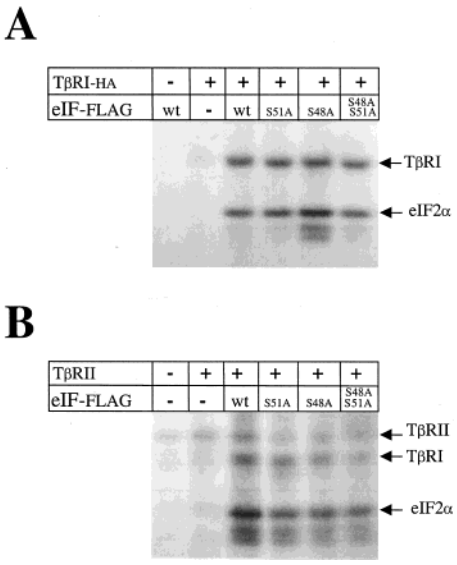

FIGURE 4: Phosphorylation of eIF2α. (A) In vitro phosphorylation of eIF2α by TβRI. Immunoprecipitated TβRI and eIF2α were subjected to in vitro kinase reactions and ^{32}P-labeled proteins were separated by SDS−PAGE. TβRI is autophosphorylated. Wild type (wt) eIF2α is phosphorylated in the presence of TβRI, but not in its absence. The S51A, S48A, and double mutant (S48AS51A) were phosphorylated by TβRI. (B) In vitro phosphorylation of eIF2α by TβRII. Immunoprecipitation of TβRII resulted in coprecipitation of endogenous TβRI, and both receptors are autophosphorylated. EIF2α wild type and mutant constructs were phosphorylated when coprecipitated with the TGFβ receptors. Immunoprecipitation of endogenous TβRII using anti-TβRII was observed in untransfected control cells (first lane).

To determine whether eIF2α plays any functional role in TGFβ signaling, we performed studies with the TGFβ-responsive reporter plasmid p3TP-Lux. p3TP-Lux contains elements from the plasminogen activator inhibitor-1 (PAI-1) promoter and drives expression of a luciferase reporter gene (*23*). Transient transfection assays were performed in mink lung epithelial cells, to assess the effect of eIF2α expression on luciferase production in the presence or absence of TGFβ. EIF2α expression resulted in a decrease in luciferase activity levels measured, in response to TGFβ stimulation (Figure 5). Inhibition was not due to a general decrease in transcription, as inhibition did not occur in the absence of TGFβ, but was observed only in the presence of 100−400 pM TGFβ (Figure 5). Furthermore, luciferase levels were normalized to an internal standard (β-galactosidase expression), to control for any effects of eIF2α overexpression on luciferase translation. These results suggest that eIF2α acts as a negative regulator of TGFβ-mediated signaling in these cells.

Previous studies have shown an interaction between another eukaryotic translation initiation factor, eIF2Bα, and the intracellular domains of α_{2A}- and α_{2B}-adrenergic receptor (*29*). In more recent studies, the purification of native adrenergic receptor resulted in copurification of eIF2Bα, together with a 14-3-3 protein. It has been suggested that the cytoplasmic domains of the receptor provide a surface for the interaction of the eIF2Bα−14-3-3 complex (*30*). We have previously reported the interaction of SmRK1 and TβRI with *S. mansoni* 14-3-3ϵ and demonstrated that 14-3-3ϵ enhances signaling by TβRI in response to TGFβ (*22*). We were interested then, in determining if an interaction might occur between our TGFβ receptor-interacting eIF2α and

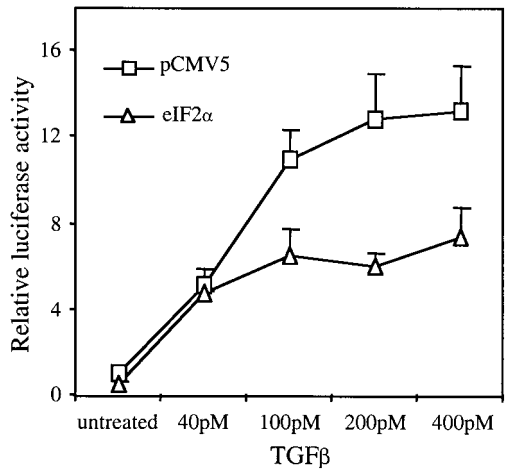

FIGURE 5: *S. mansoni* eIF2α inhibits TGF*β*-induced transcriptional activation of p3TP-Lux. Mink lung epithelial cells were transiently transfected with p3TP-Lux reporter plasmid, *β*-galactosidase construct and either empty vector (pCMV5) or pCMV2-eIF2α-FLAG (eIF2α). Cells were subsequently incubated in increasing levels of TGF*β*, and the relative luciferase activity from cell lysates was determined. Luciferase activity was normalized to *β*-galactosidase activity.

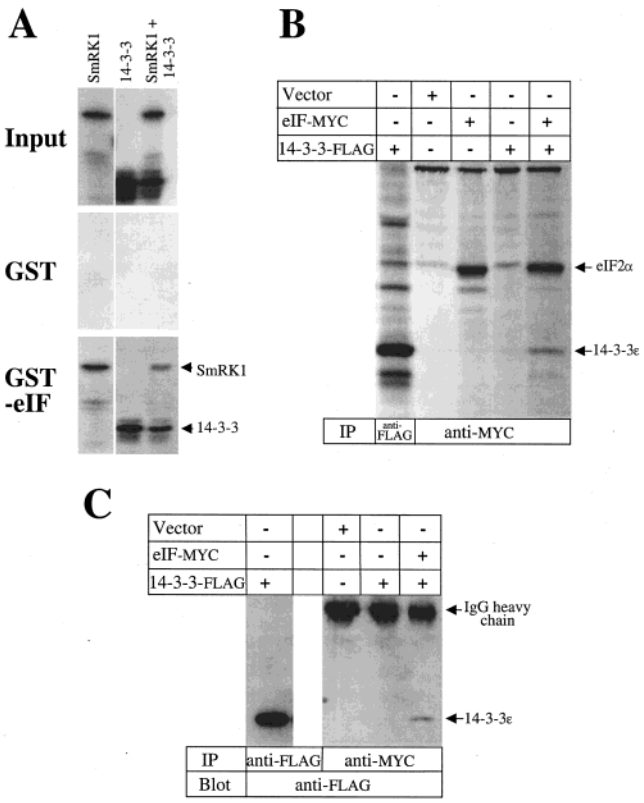

FIGURE 6: Association of eIF2α with 14-3-3*ϵ*. (A) In vitro interaction of eIF2α and 14-3-3*ϵ*. ^{35}S-labeled SmRK1 and 14-3-3*ϵ*, either alone or together were incubated with glutathione-sepharose-coupled GST (middle panel) or GST-eIF2α (bottom panel). Following washing, eluted and input (top panel) proteins were analyzed by SDS−PAGE and fluorography. Results are representative of three individual experiments. (B) In vivo association of eIF2α and 14-3-3*ϵ*. COS-7 cells were transfected with MYC-tagged eIF2α or FLAG-tagged 14-3-3*ϵ* either alone or together, or with empty vector as a control. Cells were metabolically labeled overnight, lysed, and the lysates subjected to anti-MYC or anti-FLAG immunoprecipitation (IP). Coprecipitation of 14-3-3*ϵ* was observed with an anti-MYC IP. (C) EIF2α association with 14-3-3*ϵ* detected by immunoblot. COS-7 cells were transfected with vector alone, with eIF2α-MYC alone, or in combination with FLAG-tagged 14-3-3*ϵ*. Cells were lysed and immunoprecipitated by anti-MYC or anti-FLAG antibodies. Coprecipitating 14-3-3*ϵ* was detected by immunoblot (blot) with anti-FLAG antibodies. Immunoglobulin heavy chain is indicated.

schistosome 14-3-3*ϵ*, and, whether these two proteins associate in a complex with TGF*β* receptors. First, we assessed the ability of eIF2α and 14-3-3*ϵ* to directly interact in vitro. ^{35}S-Labeled in vitro-translated 14-3-3*ϵ* interacted directly with eIF2α fused to GST, but not to GST alone (Figure 6A, first lane). To determine whether eIF2α retained the ability to bind SmRK1 in the presence of 14-3-3*ϵ*, we added both SmRK1 and 14-3-3 to GST-eIF2α. Both proteins directly interacted with GST-eIF2α, and repeat experiments demonstrated no significant difference in levels of binding of either protein (Figure 6A, second lane). To determine whether the eIF2α−14-3-3*ϵ* interaction might also occur in vivo, we assessed the ability of the two proteins to interact by coimmunoprecipitation from metabolically labeled cells. FLAG-tagged 14-3-3*ϵ* and MYC-tagged eIF2α were co-expressed in COS-7 cells. Anti-MYC immunoprecipitation of eIF2α resulted in co-immunoprecipitation of 14-3-3*ϵ* (Figure 6B). To confirm the interaction observed with metabolically labeled cells, the coimmunoprecipitation was repeated and examined by immunoblot. As before, 14-3-3*ϵ* specifically coimmunoprecipitated with eIF2α (Figure 6C).

14-3-3 proteins bind to target proteins at specific consensus binding sites, RSX*p*SXP and RXY/FX*p*SXP, where *p*S represents phosphorylated serine (*31*). As eIF2α does not contain a conserved consensus 14-3-3 binding site, we attempted to identify the region within eIF2α where 14-3-3 binds. Truncated eIF2α mutants, in which N-terminal or C-terminal regions of the protein were removed, were generated by PCR and in vitro translation and labeling, and added to GST alone or GST-14-3-3*ϵ*. Whereas, eIF2α DN, which lacked amino acids 1−115, did not associate with GST-14-3-3*ϵ* (Figure 7A, top panel), eIF2α ΔC, which lacked C-terminal amino acids 157−328, retained the ability to interact with 14-3-3*ϵ* (Figure 7A, bottom panel). These data suggest that a 14-3-3 binding site may reside within the N-terminal half of eIF2α protein. Although consensus 14-3-3 binding sites generally contain a phosphoserine residue, phosphothreonine-containing binding sites have also recently been identified (*32*). The phosphorylated residues within these sites have been shown to play a key role in the interaction of 14-3-3 with target proteins (*31*, *32*). Therefore, we aimed to determine whether phosphorylation within eIF2α was important for its interaction with 14-3-3*ϵ*. ^{35}S-Labeled eIF2α was treated with alkaline phosphatase (AP), and the ability of this dephosphorylated eIF2α to interact with GST-14-3-3*ϵ* was assessed. AP treatment of eIF2α resulted in a decrease in the level of association of eIF2α with GST-14-3-3*ϵ*, as compared to untreated eIF2α (Figure 7B). Densitometric analysis of repeat experiments showed consistently an approximately 50% reduction in binding following AP treatment (data not shown). The correlation between eIF2α dephosphorylation and reduced binding to GST-14-3-3*ϵ*, suggests that the interaction between eIF2α and 14-3-3*ϵ* is, at least in part, phosphorylation dependent.

We have previously shown that 14-3-3*ϵ* is a positive regulator of TGF*β*-mediated signaling, and in this report, we describe the inhibitory effect of eIF2α on signaling by TGF*β* receptors, as assessed by transcription from the

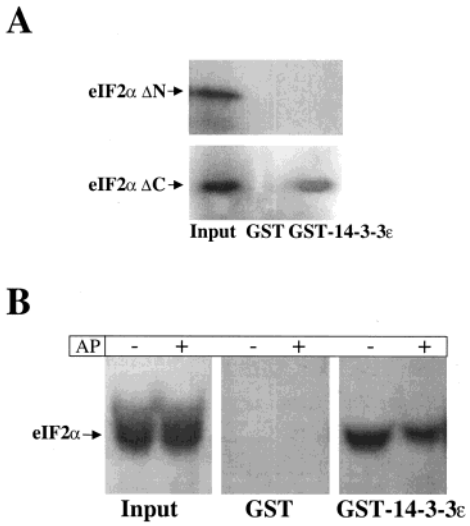

FIGURE 7: Phosphorylation-dependent interaction of eIF2α and 14-3-3ε and localization of 14-3-3 binding site on eIF2α. (A) Localization of 14-3-3ε binding site to N-terminal half of eIF2α protein. In vitro translated ^{35}S-labeled truncated eIF2α proteins eIF2αΔN (top panel) and eIF2αΔC (bottom panel) were incubated with glutathione-sepharose-coupled GST or GST-14-3-3ε; following extensive washing, bound proteins were analyzed by SDS−PAGE and fluorography. (B) Association of eIF2α and 14-3-3ε is dependent on phosphorylation of eIF2α. ^{35}S-labeled eIF2α was untreated or treated with alkaline phosphatase (AP) for 60 min, and incubated with glutathione-sepharose-coupled GST (middle panel) or GST-14-3-3ε (right panel). Following washing, eluted and input (left panel) proteins were analyzed by SDS−PAGE and fluorography. Results are representative of three individual experiments.

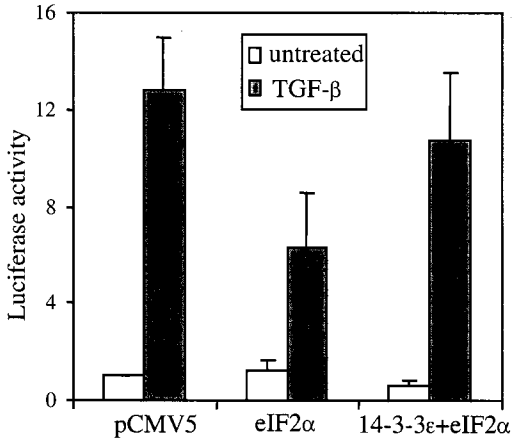

FIGURE 8: *S. mansoni* 14-3-3ε abrogates the inhibition of TGFβ-induced transcriptional activation of p3TP-Lux by eIF2α. Mink lung epithelial cells were transiently transfected with p3TP-Lux reporter plasmid, β-galactosidase construct and pCMV2-eIF2α-FLAG (eIF2α) alone or together with pCMV2-14-3-3ε-FLAG, or with empty vector (pCMV5). Cells were subsequently incubated for 20 h in the presence of 500 pM TGFβ, and the relative luciferase activity was measured in cell lysates. Luciferase activity was normalized to β-galactosidase activity.

reporter plasmid p3TP-Lux. Having also demonstrated in vivo association of these two proteins, we were interested in determining what effect coexpression of the two proteins might have on TGFβ signaling. Whereas 14-3-3ε acts to enhance signaling in R1B cells, a mutant derivative of mink lung epithelial cells, this stimulation was not observed in the nonmutated parental cell line (data not shown). As before, expression of eIF2α alone resulted in decreased luciferase production from p3TP-Lux, in response to TGFβ stimulation (Figure 8). In contrast, when eIF2α was coexpressed with 14-3-3ε, this inhibitory effect of eIF2α disappeared, and luciferase expression returned to the basal level observed in control cells, transfected with empty vector (Figure 8). Thus, 14-3-3ε appears to abrogate eIF2α's ability to inhibit signaling by TGFβ receptors, in mink lung epithelial cells.

DISCUSSION

Previously, we described the association of SmRK1 and 14-3-3ε, and demonstrated that 14-3-3ε interacts with and enhances TGFβ-mediated signaling by the human type I TGFβ receptor, TβRI (*22*). We now report the identification of *S. mansoni* eukaryotic translation initiation factor 2α subunit (eIF2α), through its interaction with SmRK1. EIF2α inhibits TGFβ-mediated signaling when expressed in mammalian cells, as measured by activation of the p3TP-Lux promoter, and is phosphorylated by both TβRI and TβRII in vitro. Strong eIF2α association is observed with kinase inactive receptors, in particular with the type II TGFβ receptor, TβRII. Furthermore, we have observed an independent interaction between eIF2α and 14-3-3ε in vivo, and shown that coexpression of 14-3-3ε abrogates eIF2α's inhibitory effect on TGFβ signaling. EIF2α, like 14-3-3ε, appears to modulate TGFβ-mediated signaling; thus, the interaction of these two proteins with each other, and with the TGFβ receptors, is likely to be important for fine-tuning the regulation of TGFβ signal transduction.

EIF2α proteins, highly conserved among eukaryotes, comprise a component of translation initiation factor eIF2. EIF2 catalyses the first regulated step of protein biosynthesis, loading of the initiator Met-tRNA$_i$ onto the 40 S ribosomal subunit, to form a ternary complex. EIF2 is composed of three nonidentical subunits, γ, β, and α, and appears to function as a stable trimer. Phosphorylation of the α subunit of eIF2 at serine 51 is catalyzed by a family of specific eIF2α kinases in response to various cellular stresses, and results in the general inhibition of translation initiation. During translation initiation, GTP complexed with eIF2 is hydrolyzed to GDP. Phosphorylation of eIF2α inhibits the function of eIF2B, preventing recycling of eIF2-GDP to the GTP bound form. This is one of the most important mechanisms for repressing global rates of protein synthesis (reviewed in ref *27*). Serine 48 of eIF2α has been shown to play a role in the high affinity interaction between eIF2 and eIF2B (*28*). Although eIF2α has a well-defined role in the regulation of protein synthesis, it had never previously been shown to interact with any membrane receptor.

While the association of initiation factors with transmembrane TGFβ receptors may seem surprising, it has previously been proposed that initiation factors are multifunctional, and perform unrelated tasks, in addition to the initiation of protein translation. The α subunit of another initiation factor, eIF2B, interacts with the intracellular domains of α_{2A}- and α_{2B}-adrenergic receptor (*29*). In this case, overexpression of the initiation factor causes a slight but significant enhancement of receptor-mediated signaling. Also, the p48 subunit of eIF3 functions not only as a component of the eIF3 complex, but also binds HTLV1-tax protein and plays a role in its cytoplasmic translocation (*33*).

A number of mammalian TGFβ receptor-interacting proteins have been identified by yeast two-hybrid screens.

One of particular interest is TRIP-1, shown to interact with TβRII (*14*). TRIP-1 shares 100% identity with p36, a subunit of eIF3 complex (*34*). It interacts specifically with TβRII and also associates with the heteromeric TGFβ complex. Whereas, we have shown that eIF2α interacts most strongly with kinase inactive TβRII, the interaction between TRIP-1 and TβRII is dependent on the kinase activity of the receptor (*34*). Despite this difference, the association of the two initiation factors with TβRII shares common features; both proteins are phosphorylated by TβRII, and expression of eIF2α or TRIP-1 results in inhibition of TGFβ-mediated signaling, as measured by transcription from the PAI-1 promoter (*14*, *34*).

EIF2α was identified in the yeast two-hybrid screen, through its interaction with the intracellular domain of SmRK1, which we previously described as a divergent type I receptor (*3*). In more recent studies, we have shown that SmRK1 can bind and signal in response to human TGFβ (*20*). We suggest that SmRK1, like *Caenorhabditis elegans* Daf-1, may signal in the absence of a type II receptor and may represent an evolutionary remnant, which existed prior to the divergence of type I and type II receptors, and thus, may contain features of both receptors (*20*, *35*). Whereas eIF2α interacts equally well with both TβRI and TβRII in vitro, both interactions appear stronger following dephosphorylation of the receptors. In in vivo studies, a strong association with TβRII, and a much weaker association with TβRI was observed, and here, eIF2α interaction was only observed with kinase inactive receptors. Through a recently discovered sequencing error, we now know that the SmRK1 construct used in the yeast two-hybrid assay was incomplete, and as a result was also kinase dead. This use of signaling deficient SmRK1 in the two-hybrid assay likely allowed the interaction between SmRK1 and eIF2α to be detected.

EIF2α may play an inhibitory role in TGFβ receptor signaling. Our results indicate that increased eIF2α expression results in a decreased TGFβ response, as assessed by transcription from PAI-1. The mechanism by which eIF2α inhibits the PAI-1 response is as yet unclear. Other TGFβ receptor-interacting proteins that similarily affect TGFβ signaling include, the eIF3 component TRIP-1, BαPP2A, and STRAP (*13*, *16*, *34*). Recently, a mechanism for inhibition by STRAP, which interacts with both TβRI and TβRII, has been determined. STRAP was shown to synergize with the antagonistic Smad7, through recruitment and stabilization of Smad 7 with active TβRI, to inhibit the TGFβ-induced transcriptional response (*36*). It will be of interest to determine whether this inhibition of signaling occurs through the classical Smad pathway or through a Smad-independent pathway. Preliminary results show that eIF2α does interact with both schistosome Smad proteins in vitro (data not shown). However, Smad cotransfection studies and experiments with a range of reporter constructs will be required to determine the precise downstream signaling pathway influenced by eIF2α.

In addition to its interaction with TGFβ receptors, we have also demonstrated an interaction between eIF2α and 14-3-3ϵ. 14-3-3 proteins interact with many key signaling molecules to regulate intracellular signal transduction events and cell cycle progression (*37*). Previously, we have reported that 14-3-3ϵ binds to and enhances signaling from TβRI in R1B cells (*22*). We now show that 14-3-3ϵ and eIF2α interact both in vitro and in vivo, and that coexpression of 14-3-3ϵ abrogates the inhibitory effect of eIF2α on TGFβ signaling. 14-3-3 proteins have been shown to bind phosphoserine residues with the consensus binding sites RSX*p*SXP and RXY/FX*p*SXP in many target proteins (*31*). However, a number of nonconsensus binding sites, some of which include a phosphothreonine residue, have also been reported (*31*, *32*). EIF2α contains neither a consensus motif nor any other previously identified 14-3-3 binding sites; therefore, a truncation study was performed to localize 14-3-3 binding. A 14-3-3 binding site was localized to the N-terminal half of the protein. This half of the eIF2α protein contains many regions of sequence identity, conserved among the invertebrate and mammalian sequences, and includes a number of conserved serine residues. To confirm that phosphorylated residues are involved in the eIF2α-14-3-3ϵ interaction, the ability of alkaline phosphatase treated eIF2α to interact with 14-3-3ϵ was ascertained. Concomitant with the dephosphorylation of eIF2α, a strong reduction in the amount of eIF2α bound by 14-3-3ϵ was observed, demonstrating that the interaction between the two proteins is, at least in part, phosphorylation dependent.

EIF2α binds both SmRK1 and 14-3-3ϵ simultaneously in vitro, and binding of one protein does not preclude binding of the other, suggesting that binding to eIF2α by 14-3-3ϵ, and the receptor occurs at different regions on the protein. We have also demonstrated that eIF2α−14-3-3ϵ binding is phosphorylation-dependent, whereas the interaction between eIF2α and TGFβ receptors appears stronger in the absence of phosphorylation. The observation that eIF2α associates more strongly with receptors that are not actively signaling leads us to propose that activation of the receptor causes a conformational change in eIF2α, such that it dissociates from the receptor. This change in confirmation may result from phosphorylation, and we have demonstrated that eIF2α is phosphorylated in vitro, by both TβRI and TβRII. Since phosphorylation occurs at sites other than serine 48 and 51, an intriguing possibility is that phosphorylation of eIF2α by the TGFβ receptors may create the phosphorylated 14-3-3ϵ binding site, allowing the eIF2α−14-3-3ϵ association to occur. Further mutational analysis will be necessary to identify the phosphorylation site and to determine whether this is also the site for 14-3-3ϵ binding. The significance of the enhanced autophosphorylation of both receptors observed when coexpressed with eIF2α also remains to be determined.

An interaction between the z isoform of 14-3-3 and the third intracellular loop of $a_2$adrenergic receptors has been described (*30*). The intracellular domain of the receptor also associates with the initiation factor component eIF2Bα (*29*). Purification of native receptor results in copurification of both 14-3-3z and eIF2Bα (*30*). It has been proposed that the receptor provides a surface for the interaction of the 14-3-3ζ−eIF2Bα complex, which is likely to influence signaling by the G-protein-coupled receptor (*30*). Several attempts to demonstrate the existence of a complex between the TGFβ receptors, 14-3-3ϵ, and eIF2α proved unsuccessful, perhaps because the interactions are of too low affinity to persist in the immunoprecipitation procedures or because they are too transient in nature.

Whereas 14-3-3ϵ enhances TGFβ signaling, and eIF2α appears to antagonize signaling, expression of the two proteins together restores signaling to basal levels. These data

together with our interaction studies suggest that the two proteins act as modulators of TGFβ receptor signaling, and their relative expression levels may play an important role in determining the outcome of the TGFβ signaling response.

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