Expression of Functional Schistosoma mansoni Smad4

ROLE IN ERK-MEDIATED TRANSFORMING GROWTH FACTOR β (TGF-β) DOWN-REGULATION*†

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Ahmed Osman, Edward G. Niles, and Philip T. LoVerde‡

From the Department of Microbiology and Immunology and Witebsky Center for Microbial Pathogenesis and Immunology, School of Medicine, State University of New York, Buffalo, New York 14214

Members of the transforming growth factor (TGF)-β superfamily play pivotal roles in cell migration, differentiation, adhesion, pattern formation, and apoptosis. The family of Smad proteins acts as intracellular signal transducers of TGF-β and related peptides. Smad4, a common mediator Smad (co-Smad), performs a central role in transmitting signals from TGF-β and activins. Schistosoma mansoni receptor-regulated Smad1 and Smad2 were previously identified and shown to act in TGF-β signaling. Herein, we report the identification and characterization of a Smad4 homologue from S. mansoni and provide details about its role in mediation and down-regulation of TGF-β signaling in schistosomes. In order to identify the schistosome co-Smad, we designed degenerate primers based on the sequence of the conserved MHI/MHII domains of Smad4 proteins, which were used in PCR to amplify a 137-bp PCR product. A S. mansoni adult worm pair cDNA library was screened resulting in the isolation of a cDNA clone that encodes a 738 amino acid protein (SmMad4). SmMad4 was shown to interact with schistosome R-Smads (SmMad1 and SmMad2) in vivo and in vitro. The interaction with SmMad2 was dependent on the receptor-mediated phosphorylation of SmMad2. In addition, several potential phosphorylation sites for Erk2/ERK kinases were identified in the SmMad4 linker region and shown to be phosphorylated in vitro by an active mutant of mammalian Erk2. Furthermore, Erk-mediated phosphorylation of SmMad4 decreased its interaction with the receptor-activated form of SmMad2, in vitro. SmMad4 was shown to complement a human Smad4 deficiency through the restoration of TGF-β responsiveness in MDA-MB-468 breast cancer cells.

Helminths of the genus Schistosoma are the causative agents of schistosomiasis, an endemic tropical disease affecting about 200 million people worldwide, which is a major cause of morbidity (www.who.int/tdr/diseases/schisto/diseaseinfo.htm). It is generally thought that the schistosome receives host signals that are utilized in parasite development. Thus, host-parasite interactions are likely to have co-evolved and to be selective for both the intermediate and the definitive host (1, 2). In the definitive vertebrate host, the parasite migrates from the site of infection to the final destination in a complex journey that proceeds with concomitant development and maturation, likely guided by host factors (3, 4). Sequence, female worm sexual maturation and subsequent egg production, the major cause of the pathological consequences of schistosomiasis, is induced by mating with male worms. Female sexual development is likely to be stimulated by a set of chemical and/or mechanical signals from the male (5). Therefore, an investigation of signal transduction pathways in schistosomes may permit us to understand the mechanisms by which the parasite receives and responds to both self- and host-derived signals. Such studies will yield insights into the host-parasite relationship and male-induced female maturation.

Prior investigations identified growth factor receptors and orthologues of components of signaling pathways in schistosomes (6–12). Transforming growth factor β (TGF-β)† is an example of a signaling pathway that affects a wide variety of cell types and regulates different vital processes such as cell growth, differentiation, morphogenesis, and apoptosis (13–15). The intracellular signal transmission in TGF-β pathway is initiated through ligand-induced formation and activation of a heteromeric receptor complex of type II and type I serine/threonine kinase receptors located at the cell surface. This process involves the binding of a TGF-β ligand to the constitutively phosphorylated type II kinase receptor, which triggers the interaction with and phosphorylation and activation of the corresponding type I receptor (16). The activated receptor complex in turn relays the signal to the downstream member, the receptor-regulated Smad (R-Smad). Activation of R-Smads occurs via direct phosphorylation of the two most C-terminal serine residues ((T/S)XSXS motif) by the specific type I receptor (17, 18). R-Smads are recruited to different activated receptor complexes, depending on the activating ligand. Smad1, Smad5, and Smad8 are activated and phosphorylated by receptors of the bone morphogenetic protein (BMP) subfamily (18, 19); while Smad2 and Smad3 receive the signal relayed from activated receptors of the TGF-β and activin subfamilies (17, 19, 22, 23). Upon activation by the receptor complex, R-Smad forms a hetero-oligomeric complex with a common Smad (co-Smad). The co-Smad, called Smad4 in vertebrates, or Medea in Drosophila, acts as a shared partner for both BMP-specific and TGF-β/activins-specific R-Smads (24, 25). Smad4 was origi-
nally identified as a tumor suppressor gene that is deleted or mutated in pancreatic carcinomas, and is also known as DPC4 (deleted in pancreatic carcinoma locus 4) (26, 27). Although Smad4 shares many sequence similarities with R-Smads, it lacks the phosphorylation signature located at the C-terminal end of the MH2 domain of R-Smads and thus neither associates with the receptor complex nor acts as a substrate for receptor phosphorylation (17, 28). The newly formed Smad4/R-Smad complex then translocates to the nucleus where it binds to promoter sequences and modulates the transcription of a subset of responsive genes required to exert the specific TGF-β ligand effect (29, 30). In addition to the receptor complex and the corresponding R-Smad, signal specificity is determined by the nuclear partner that binds and directs Smad4/R-Smad complex to the target gene(s). Smad proteins are considered crucial members in the TGF-β signaling since they receive the signal from the activated receptor complex at the cell surface and relay it to the nucleus where the specific outcome/phenotype will be orchestrated.

Structurally, R-Smads and co-Smad consist of 2 conserved domains, located at the N and C termini of the proteins, called Mad homology domains 1 and 2 (MH1 and MH2), respectively, linked by a highly variable, proline-rich linker region (31). The MH1 domain mediates DNA binding (32–34) and negatively regulates the functions of the MH2 domain (35). The MH1 domain also contains a cluster of basic residues often followed by hydrophobic aliphatic and acidic residues, which serve as a nuclear localization signal (NLS) (36–38). The MH2 domain is responsible for transcription activation (21, 39, 40) and interactions with other Smads, receptor kinases, and/or nuclear partners (41–43). The linker region of R-Smads contains PX/S/T/P phosphorylation motifs, the consensual phosphorylation site for the Ras-activated mitogen-activated protein kinases Erk1 and Erk2 (44). Erk phosphorylation results in blocking the nuclear accumulation of R-Smads and consequently down-regulating TGF-β signaling (45–47).

The N-terminal end of the linker region also contains a leucine-rich nuclear export signal (NES), which mediates the nuclear export of Smad4. The C terminus of the linker region of Smad4 contains a proline-rich transcription activation domain (Smad4 activation domain; SAD) that is not only necessary but also sufficient to activate maximal Smad-dependent transcriptional responses (35, 48, 49).

Recent studies showed that schistosomes contain several members of the TGF-β pathway. A TGF-β type 1 receptor homologue, SmRkr1 and called SmTβR-I thereafter was the first member to be identified and shown to be localized to the surface of the parasite (8). The identification of the schistosome type I receptor spurred the search for other members of TGF-β pathways in the parasite *S. mansoni*, which resulted in the identification of 2 members of R-Smad subfamily, SmSmad1 (10) and SmSmad2 (10, 11). Interestingly, SmSmad2 was shown to interact with and act downstream of SmTβR-I, where it serves as a substrate for receptor phosphorylation and translocates into the nucleus of mammalian cells after induction with human TGF-β (10, 11). The above data suggest the presence of a common Smad homologue in schistosomes and underscores the significance of pursuing the search for Smad4. In this study, we report the identification and characterization of SmSmad4, the common Smad homologue of *S. mansoni*, and we demonstrate its interaction with schistosome R-Smads.

### EXPERIMENTAL PROCEDURES

**Identification and Isolation of SmSmad4 cDNA**—Protein sequences of Smad4 homologues were aligned using Pileup (Genetics Computer Group, GCG-Wisconsin Package, version 9.1) to define areas of high conservation in the MH1 and MH2 domains. Degenerate primers were synthesized, based on the amino acid sequence of the homologous regions, and were employed in different combinations in PCR using *S. mansoni* adult worm pair cDNA. One primer pair spanning a region unambiguously containing the MH1 domain (Fig. 1A) amplified a 137-bp PCR product. Primers F3 and R4 (5′-CCNCAYGTNRNTAYGGCNMGHTNGMTGCGG3′-3′-5′-CANACNCYTCTCRTARTGNGRTT-3′, respectively). Based on the DNA sequence of the 137-bp product, two specific primers (DPC-fwd and rev; bp 412–435 as forward primer and the complementary sequence of bp 467–489 as reverse primer, respectively), were designed downstream of F3 and R4 primer sequences and amplified a 78-bp-specific cDNA product. The later DNA fragment was radiolabeled with [α-32P]dCTP using MegaPrime random priming labeling kit (Amersham Biosciences) and used to screen 400,000 plaques from a non- amplified *S. mansoni* adult worm pair λZAPII cDNA library.

**Cloning and Expression of SmSmad4**—DNA and protein sequence analysis programs were used in the GCG-Wisconsin Package, version 9.1. Sequence analysis of the SmSmad4 cDNA revealed the presence of a unique BglII site 742-bp downstream of the vector EcoRI cloning site. A 5′-primer (bp 47–72), in which the C was replaced by an A creating an EcoRI site upstream of the start ATG codon, was used as a forward primer along with a reverse primer (complementary sequence of bp 792–774) to amplify a 728-bp product. The PCR product was cloned in pcR2.1-TOPO cloning vector (Invitrogen), sequenced to confirm the absence of any PCR-generated errors, digested with EcoRI and BglII and recloned into the parental pSmSmad4-BlueScript-SK+ vector to generate a modified version of the cDNA clone in which the sequence upstream of the start ATG codon was removed and an EcoRI site was inserted just before and in-frame with the start codon. The modified vector, pSmSmad4-cod-BlueScript-SK+, was digested with EcoRI and Xhol to yield a SmSmad4 DNA fragment suitable for cloning in several vectors including pGEX-4T-1 (Amersham Biosciences; digested with EcoRI-Xhol), pMAL-c2x (New England Biolabs; digested with EcoRI-Sall) for prokaryotic expression; pGAL4Δ-2.1 (GAL4-activation domain vector, Stratagene; digested with EcoRI-Xhol), pBDGAL4-cam (GAL4-DNA binding domain vector, Stratagene; digested with EcoRI-Sall) for yeast two-hybrid analysis, pcDNA3.1-His (Invitrogen; digested with EcoRI-Xhol) for expression in mammalian cells, and pCITE-4a (Novagen; digested with EcoRI-Xhol) for in vitro transcription/translation (see Table S1, online Supplemental Data).

**Production of Anti-SmSmad4-specific Antiserum**—In addition to the full-length constructs, a DNA fragment encoding the SmSmad4 linker region (bp 401–1183; 216 amino acids) was also amplified, cloned in pcR2.1-TOPO, sequenced and cloned in pMAL-c2x vector for expression of a fusion protein that shares no homology to other schistosome Smad proteins. MBP-SmSmad4-Linker fusion protein was used to immunize a New Zealand rabbit and BALB/c mice. A dose of 200 μg of the fusion protein in Freund’s complete adjuvant was used to immunize the rabbit as a primary dose and 200 μg in Freund’s incomplete adjuvant were used for 2 booster doses at 4-week intervals. An activating dose (200 μg in 1× phosphate-buffered saline) was used 7 days prior to sacrificing the animal. Mice were immunized with the same reagents following same time frame (50 μg of each dose). Serum for SmSmad4 and IgG fractions were affinity-purified over protein A-Sepharose resin (Amersham Biosciences) and quantified. Preimmune rabbit and mice sera were processed similarly to provide reagents for negative controls. Affinity-purified IgG was used for immunoprecipitation, immunofluorescence, and Western blot analyses.

**Western Blotting and Immunofluorescence**—Protein extracts were prepared from *S. mansoni* adult worm pairs as previously described (11). 20 μg were loaded per lane on 4–12% gradient SDS-polyacrylamide gels (Invitrogen) and size-separated. SDS gels were blotted onto polyvinylidene difluoride membranes (Immobilon P; Millipore), and the blots were probed with 0.75 μg/ml of either preimmune IgG or anti-SmSmad4 IgG. Biotinylated secondary antibody reagents (goat anti-rabbit and goat anti-mouse IgG, 0.75 μg/ml; Molecular Probes, Inc.) were used to probe the reactive primary antibodies and the immune complexes were detected by alkaline phosphatase-conjugated streptavidin (1 μM; Molecular Probes, Inc.) and alkaline phosphatase substrate kit (NBT/BCIP Vector Kit, Vector Laboratories).

**Immunofluorescence** was also employed to visualize the native SmSmad4 in adult worm cryosections, probed with biotinylated secondary antibody reagents (5 μg/ml; Molecular Probes, Inc.) and detected with AlexaFluor 647-conjugated streptavidin (5 μg/ml; Molecular Probes, Inc.). AlexaFluor 647 is a far-red fluorochrome that emits at a maximum wavelength of 647 nm. At this wavelength, auto-fluorescence produced by phenolic compounds in schistosome sections would not be visualized. Probed sections were evaluated using a Bio-Rad MRC-1024 confocal microscope equipped with Krypton-Argon laser and 522 nm and 680 nm filters.

**RT-PCR Analysis**—Analysis of SmSmad4 mRNA levels in different
and reverse primers, respectively, yielding a 378-bp PCR product) were reverse transcribed using a random hexamer, and SuperScript Reverse Transcriptase II (Stratagene; Invitrogen) following the vendor's recommended conditions. Reverse-transcribed cDNA samples were used as templates in PCR reactions using specific primer pairs. PCR reactions were separated by electrophoresis in 2% agarose gels, ethidium bromide-stained, and analyzed using a gel-documentation system (GelDoc1000; Bio-Rad) and quantified using Quantity One software (version 4.2.3; Bio-Rad). Control PCR reactions using reverse transcription reaction mix lacking RT enzyme were also performed to ensure the efficiency of the treatment. The primer pairs used for the yeast three-hybrid assay and a positive interaction was assessed by activation of HIS3 and ADE2 reporter genes, permitting growth on the selective medium S.D. -Leu, -Trp, -His, -Uracl (Uracl) reporter gene yielded blue colonies in the LacZ filter- (-) lift assay. The yeast strains 64/99-4a (11) and YRG2 (Stratagene) were used for the yeast three-hybrid assay. All yeast strains were obtained from the suppliers (BD Biosciences-Clontech and Stratagene).

(iii) In Vitro Interaction, GST Pull-down, and Co-immunoprecipitation—R-SmSmads/SmSmad4 interactions were evaluated by immuno-
precipitation in the presence or absence of wild type or constitutively activated SmTβRI. The first step was to assess the interaction status of the 2 forms of SmTβRI with SmSmads. S-tagged, unlabeled SmTβRI-I-wt or SmTβRI-I-QD (10 μl) was allowed to interact with 35S-labeled, non-S-tagged SmSmads (5 μl) for 1 h at room temperature, and coprecipitation was precipitated by the gel filtration (10 μl; Novagen). Protein-bound beads were washed, resuspended into SDS gel loading buffer, and size-separated in 4–12% gradient SDS-polyacrylamide gels. The gels were treated with Amplify (Amersham Biosciences), dried, and exposed to x-ray film.

GST pull-down assays were performed to evaluate the interaction of SmSmad4 with SmSmad2, as described previously (11). Briefly, [35S]-SmSmad4 was translated, unlabeled SmTβRII-chem was used to evaluate the ability of Erk kinase to phosphorylate MBP-SmSmad4-Linker rabbit IgG (3 μg) was added, and incubation was extended for an additional 30 min at room temperature. Protein complexes were precipitated with rProtein A Sepharose beads (Amersham Biosciences, 20 μl). The reactive beads were washed and processed as described above. GST pull-down experiments as described above. GST pull-down experiments with GST-SmSmad2-Linker-bound beads were similarly processed to serve as negative controls.

Co-immunoprecipitation assays were conducted using unlabeled in vitro translated SmSmad4 (10 μl), with [35S]-SmSmad2-MH2, SmSmad2-MH2/AAA or full-length SmSmad1 (5 μl), in the presence or absence of SmTβRI-wt or SmTβRI-QD (10 μl). Reactions were incubated for 1 h at room temperature, then anti-MBP-SmSmad4-Linker rabbit IgG (3 μg) was added, and incubation was extended for an additional 30 min at room temperature. Protein complexes were precipitated with rProtein A Sepharose beads (Amersham Biosciences, 20 μl). The reactive beads were washed and processed as described above.

(i) In Vivo Interaction, Yeast Two-hybrid and Three-hybrid Assays—In the yeast two-hybrid assays, yeast host strains AH109 (BD Biosciences-Clontech) and YRG2 (Stratagene) were used. The positive interactions were judged by activation of HIS3/ADE2 reporter genes for AH109, as determined by the ability of transformed cells to grow on selective medium (synthetic-dextrose; S.D.) lacking leucine, trypto-
phane, histidine, and adenine in the presence of 2.5 mm 3-amin 1,2,4 triazole (an inhibitor of HIS3 gene) in the host cells; 3-AT (S.D. -Leu, -Trp, -His, -Ade, 2.5 mm 3-AT). Positive interactions in YRG2 cells were assessed based on the activation of HIS3 reporter gene, as indicated by growth on the selective medium S.D. -Leu, -Trp, -His. In both strains, when activated, the LacZ reporter gene yielded blue colonies in the LacZ colony- (-) lift assay. The yeast strains 64/99-4a (11) and Y187 (Stratagene) were used for the yeast three-hybrid assay. Activation of the reporter genes was assessed in absence of uracil. Activation of the reporter genes was assessed in absence of or presence of uracil to provide a comparative measure to determine the effect of the expression of the third protein on the interaction of the GAL4-AD and -BD fusion proteins. The degree of interaction was quan-
tified by performing liquid LacZ assay on the transformed colonies of both strains, in addition to growth on selective medium (synthetic-dextrose, S.D. -Leu, -Trp, -Ura) for yeast strain Y187.

(ii) In Vivo Interaction, Yeast Two-hybrid and Three-hybrid Assays—In the yeast two-hybrid assays, yeast host strains AH109 (BD Biosciences-Clontech) and YRG2 (Stratagene) were used. The positive interactions were judged by activation of HIS3/ADE2 reporter genes for AH109, as determined by the ability of transformed cells to grow on selective medium (synthetic-dextrose; S.D.) lacking leucine, trypto-
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sponsiveness of SmSmad4 to human TGF-β ligands, SmSmad4 was cloned in the mammalian expression vector, pcDNA3.1-His (Invitrogen). Mink lung epithelial cells, Mv1Lu (ATCC CCL-64) and the Smad4-deficient human breast cancer cell line, MDA-MB-468 (ATCC HTB-132) were used and maintained according to ATCC recommended instructions. Double CsCl-purified pSmSmad4-cDNA-His was used to transfect the two cell lines along with the TGF-β-responsive reporter plasmid vector, p800-Luc, (kindly provided by Dr. D. Leskutofb) in which 800 bp of the promoter region of the TGF-β-positively regulated gene, plasmogen activator inhibitor 1 (PAI-1) was cloned upstream of firefly luciferase reporter gene (52). SmSmad4 was also transfected along with the pCAL2 reporter vector (Kindly provided by Dr. Rick Derynck), which contains the luciferase reporter gene under control of the human cyclo A promoter, a TGF-β-negatively regulated gene (53). LipofectAMINE 2000 reagent (Invitrogen) was used to transfect the mammalian cells following the recommended manufacturer’s instructions. Transfected cells were incubated with transfection mix, in triplicate, after 6 h, the mix was changed to complete culture media containing 10% fetal bovine serum and either left untreated or treated with 0.5 nM TGF-β (rhTGF-β1; R&D Systems) for 4 h. Cells were washed, harvested in lysis buffer, and assayed for luciferase activity. The dual luciferase assay kit (Promega) was used to determine the expression of sea pansy (Promega), which uses the immediate early CMV promoter to drive firefly luciferase reporter gene (52). SmSmad4 was also transfected along with the pCAL2 reporter vector (Kindly provided by Dr. Rick Derynck), which contains the luciferase reporter gene under control of the human cyclo A promoter, a TGF-β-negatively regulated gene (53). LipofectAMINE 2000 reagent (Invitrogen) was used to transfect the mammalian cells following the recommended manufacturer’s instructions. Transfected cells were incubated with transfection mix, in triplicate, after 6 h, the mix was changed to complete culture media containing 10% fetal bovine serum and either left untreated or treated with 0.5% fetal bovine serum and incubated for an additional 18 h. The cells were serum-deprived overnight in culture media containing 10% fetal bovine serum and either left untreated or treated with 0.5 nM TGF-β (rhTGF-β1; R&D Systems) for 4 h. Cells were washed, harvested in lysis buffer, and assayed for luciferase activity. The constitutively expressed reporter construct, pRL-CMV (Promega), which uses the immediate early CMV promoter to drive the expression of sea pansy (Renilla reniformis) luciferase gene, was co-transfected in all cells to serve as an internal control for normalizing the data. Transfected cells were incubated with transfection mix, in triplicate, after 6 h, the mix was changed to complete culture media containing 10% fetal bovine serum and either left untreated or treated with 0.5 nM TGF-β (rhTGF-β1; R&D Systems) for 4 h. Cells were washed, harvested in lysis buffer, and assayed for luciferase activity. The dual luciferase assay kit (Promega) was used to determine the luciferase activities in transfected cells following the manufacturer’s instructions employing an Orion MFL2 microplate luminometer (Berthold Detection Systems) to measure the luminescence in transfected samples.

RESULTS

Identification and Isolation of SmSmad4 cDNA—Mammalian homology regions (MH1 and MH2) of Smad4 homologues from different species, were aligned to define areas of high conservation. Several degenerate primers were designed and used in PCR with S. mansoni adult worm cDNA yielding a PCR product that shared homology to Smad4. A primer located downstream of the original degenerate primers amplified a 78-bp DNA fragment, which was used as a probe to screen an 800-bp DNA fragment, which was used as a probe to screen an 800-bp DNA fragment, which was used as a probe to screen an 800-bp DNA fragment, which was used as a probe to screen an 800-bp DNA fragment, which was used as a probe to screen an 800-bp DNA fragment, which was used as a probe to screen an 800-bp DNA fragment, which was used as a probe to screen an 800-bp DNA fragment, which was used as a probe to screen an 800-bp DNA fragment, which was used as a probe to screen an 800-bp DNA fragment, which was used as a probe to screen an 800-bp DNA fragment, which was used as a probe to screen an 800-bp DNA fragment, which was used as a probe to screen a

| Homologue                  | SmSmad4-MH1 | SmSmad4-MH2 |
|---------------------------|-------------|-------------|
|                           | Identity    | Homology score | Identity | Homology score |
|                            | %           | %            | %        | %            |
| Drosophila melanogaster Medea | 70          | 85           | 215      | 50           | 58           | 307          |
| Drosophila melanogaster Medea-A | 70          | 85           | 212      | 50           | 58           | 306          |
| Drosophila melanogaster Medea-B | 70          | 85           | 215      | 50           | 58           | 307          |
| Homo sapiens Smad4         | 68          | 81           | 194      | 48           | 61           | 317          |
| Sus scrofa Smad4           | 70          | 87           | 194      | 51           | 64           | 320          |
| Mus musculus Smad4         | 70          | 87           | 194      | 51           | 64           | 320          |
| Rattus norvegicus Smad4    | 70          | 87           | 193      | 50           | 62           | 314          |
| Xenopus laevis Smad4A      | 70          | 87           | 193      | 50           | 63           | 311          |
| Xenopus laevis Smad4B      | 68          | 82           | 184      | 61           | 74           | 300          |
| Xenopus laevis Smad10      | 68          | 82           | 183      | 61           | 74           | 301          |
| Caenorhabditis elegans Sma4 | 49          | 69           | 156      | 40           | 55           | 156          |

Sequences producing significant alignment with SmSmad4 MH1 and MH2 domains are shown with % identity, % similarity, and homology score. Boldface values represent the highest homology hits scored against SmSmad4-MH1 and MH2 domains, which takes into account the extent of the overlap.

The results of an NCBI BLASTP search of SmSmad4 amino acid sequence are presented in Table 1. Analysis of the protein sequence revealed the conservation of the Smad4 structural features, including the NLS and DNA-binding motif in the MH1 domain and the NES in the N-terminal end of the linker region (Fig. 1A). The NLS as well as the NES are well conserved in SmSmad4 with the retention of all basic and leucine residues constituting the cores of the NLS and NES domains, respectively (Fig. 1A). The corresponding SAD sequence in SmSmad4 (Fig. 1B) although showing very little sequence conservation, retains a proline-rich pattern. Interestingly, the SmSmad4 linker region was found to contain 3 Erk1/2 phosphorylation motifs (PX/S/T/P) (Fig. 1B) compared with one or two motifs present in vertebrate homologues and none in Drosophila Medea. None of schistosome R-Smads, SmSmad1 (GenBank™ accession no. AP215933), SmSmad2 (GenBank™ accession no. APF23025), and the recently identified Smad8/9 homologue (54) contain Erk phosphorylation motifs.

Western Blotting and Immunohistochemistry—Affinity-purified IgG fractions of mice and rabbit sera, raised against the SmSmad4 linker region, were used to detect the native protein in extracts and in cryosections of adult worms. The apparent molecular size of the native SmSmad4 is ~78 kDa as determined by SDS-polyacrylamide gel and Western blot analyses (data not shown). The native protein was localized to the epithelial tissues surrounding the gut and vitelline lobules in female worms (Fig. 2E) and in the subtegmental tissues and muscle layers of male worms (Fig. 2G). IgG fractions of preimmune sera were used as a negative control (Fig. 2, A–C). As shown in the negative control sections, auto-fluorescence, observed in females using a 522-nm filter, attributed to the photonic compounds present in vitelline cells (Fig. 2B) was not observed at the far-red wavelength (680-nm filter) used to visualize the AlexaFluor-647 streptavidin conjugated to the reactive antigens (Fig. 2C).

RT-PCR Analysis—SmSmad4 mRNA levels were determined in different stages in the parasite life cycle comprising both mammalian and molluscan hosts. Compared with α-tubulin mRNA, SmSmad4 mRNA levels exhibited little variation throughout development of the parasite (Fig. 3C and Fig. 4A) including sexually immature parasites (single-sex female and male worms) (Fig. 3, B and C, lanes 16–18, and Fig. 4, A and B, lanes 7 and 8). In contrast, in the infected snail stage, which represents secondary sporocysts and to some extent the free-living infective cercarial stage, SmSmad4 showed a relatively
FIG. 1. Multiple sequence alignment of SmSmad4 and other Smad4 homologues from different species. A, pileup of MH1 domain (amino acids 37–156) and the N-terminal sequence of the linker region. Boxed sequences represent conserved motifs; NLS, nuclear localization signal, DNA binding motif, and NES, nuclear export signal. Black- and gray-boxed text represent amino acid sequence used to design DPC4/F3 and R4 degenerate primers, respectively. B, pileup of the C-terminal sequence of the linker region and the MH2 domain (amino acids 460–695). Smad4 activation domain (SAD) is shown between brackets with proline residues typed in bold face. Sequences used in these analyses are S. mansoni SmSmad4 (GenBankTM accession no. AY371484), Drosophila melanogaster Dm-Medea-B (GenBankTM accession no. AAC35436), D. melanogaster Dm-Medea (GenBankTM accession no. NP_733438), D. melanogaster Dm-MEDEA (GenBankTM accession no. AAC25634), D. melanogaster Dm-Medea-A (GenBankTM accession no. AAC35437), Xenopus laevis Xl-XSmad4A (GenBankTM accession no. BAA77514), Sus scrofa Ss-Smad4 (GenBankTM accession no. Q9GKQ9), Rattus norvegicus Rn-Smad4 (GenBankTM accession no. NP_062148), Mus musculus Mm-Smad4 (GenBankTM accession no. NP_062148), Homo sapiens Hs-Smad4 (GenBankTM accession no. NP_005350), X. laevis Xl-XSmad4B (GenBankTM accession no. BAA77515), X. laevis Xl-Smad10 (GenBankTM accession no. NP_065550), X. laevis Xi-XSmad4B (GenBankTM accession no. BA77515), X. laevis Xi-Smad10 (GenBankTM accession no. AAD16879), Caenorhabditis elegans Ce-Smad4 (GenBankTM accession no. NP_049256).
low level (Fig. 3, B, lane 4). The direct interaction of SmSmad4 with SmSmad2 was assessed by the number of colonies grown under selective conditions as compared with the number of colonies grown on the control plate (–Leu, –Trp). Those colonies also produced a faint blue-green color in the LacZ assay that required incubation for up to 3 days. The MH2 domain of SmSmad2, its AAA- and linker regions which are not phosphorylatable constructs, SmSmad2–Q/D, significantly elevated SmSmad4 interaction with either wild-type full-length SmSmad2 or its wild-type MH2 domain. Neither of the non-phosphorylatable constructs, SmSmad2–Q/D and MH2–AAA, showed detectable interaction with SmSmad4 in the presence of SmTβR-I-Q/D (Table 2, right column). These results demonstrate that phosphorylation of SmSmad2 by TβR-I-Q/D stimulates binding to SmSmad4. In contrast, the SmSmad1 interaction with SmSmad4 was significantly inhibited in the presence of SmTβR-I-Q/D.

**In Vivo Interaction among Schistosome Smads**—In order to further evaluate the interaction of SmSmad4 with SmSmad1 and SmSmad2, and the effect of SmTβR-I on this interaction, an in vitro approach was designed. Initially, the interaction of SmTβR-I with SmSmads was evaluated by co-precipitation. Both the wild-type and the constitutively activated form of SmTβR-I interacted with all SmSmads, but not with SmSmad4 (Fig. 5A). Wild-type SmSmad2 and its MH2 domain interacted with SmTβR-I-wt more than SmTβR-I-Q/D (Fig. 5A, lanes 7 and 8), while SmSmad1 showed stronger interaction with SmTβR-I-Q/D (Fig. 5A, lanes 4 and 6). SmSmad2-MH2-AAA exhibited slight preference toward the Q/D form (Fig. 5A, lanes 1 and 2).

The direct interaction of SmSmad4 with SmSmad2 was evaluated by a GST pull-down assay. GST alone and the SmSmad2-linker control exhibited minimal binding with SmSmad4 in the presence or absence of SmTβR-I (Fig. 5B, panels I and II). SmSmad2-GST showed basal level of interaction with SmSmad4 (Fig. 5B, lane 9), while SmTβR-I-wt had less effect (Fig. 5B, lane 7), and SmTβR-I-Q/D (Fig. 5B, lane 8), while SmSmad1 showed stronger interaction with SmTβR-I-Q/D (Fig. 5B, lanes 4 and 6). SmSmad2-MH2-AAA exhibited slight preference toward the Q/D form (Fig. 5A, panels I and II, lanes 4 and 6).
Similar results were also observed in co-immunoprecipitation assays. SmSmad2-MH2 and its non-phosphorylatable AAA-mutant form showed a comparable basal level of interaction with SmSmad4 (Fig. 5C, panels I and II, lane 3). In the presence of SmTβR-I-Q/D, the interaction of SmSmad4 with SmSmad2-MH2 was significantly enhanced, while it exhibited a minor decrease with SmSmad2-MH2/AAA (Fig. 5C, panels I and II, lane 5). On the other hand, the addition of wild-type receptor I (SmTβR-I-wt) produced a modest change in the interaction of SmSmad4 with SmSmad2-MH2 and with MH2/AAA (Fig. 5C, panels I and II, lane 4). SmSmad1 readily interacted with SmSmad4 (Fig. 5C, panel III, lane 3). However, both forms of SmTβR-I inhibited the SmSmad1 interaction with SmSmad4 (Fig. 5C, panel III, lanes 4 and 5). This can be attributed to the observed interaction between SmSmad1 and both forms of type I receptor (Fig. 5A, lanes 9 and 10).

Erk-mediated in Vitro Phosphorylation of SmSmad4—Analysis of the SmSmad4 protein sequence revealed the presence of 3 possible Erk1/2 phosphorylation sites in the linker region and extending to beginning of the MH2 domain. MBP-fusion proteins of both full-length SmSmad4 and its linker region were tested as substrates for the activated Erk2 kinase in vitro (Fig. 6A, lanes 5 and 8). The effect of Erk2 phosphorylation on the interaction of SmSmad4 with R-Smads was also tested. Two sets of reactions were assayed in the absence (Fig. 6B, lanes 3–5) or in the presence of Erk2 (Fig. 6B, lanes 6–8). Effect of Erk2 treatment was assessed by comparing similar samples from the 2 sets. Interestingly, although Erk2 treatment has no effect on the interaction of SmSmad4 with SmSmad2 or its MH2 domain in absence of SmTβR-I (Fig. 6B, panels I and II, lanes 3 and 6), activated Erk2 significantly inhibited SmSmad4 interaction with SmSmad2-wt and SmSmad2-MH2 in the presence of the SmTβR-I-Q/D (Fig. 6B, panels I and II, lanes 5 and 8). In case of SmSmad1, Erk2 phosphorylation of SmSmad4 had little effect on SmSmad1 interaction with SmSmad4 in the presence or absence of SmTβR-I (Fig. 6B, panel III).

SmSmad4 Participates in TGF-β Signaling in Mammalian Cells—The ability of SmSmad4 to restore TGF-β responsiveness in Smad4-deficient human breast cancer cell line, MDA-MB-468 was tested. In absence of TGF-β1 treatment, MDA-MB-468 cells transfected with pCAL2 and p800-luc reporters exhibited background luciferase activity. TGF-β1 treatment of these cells resulted in a minor change in the luciferase activities, which was expected because of the TGF-β non-responsiveness of this cell line. Expression of SmSmad4 in these cells modestly decreased the luciferase activity in pCAL2-transfected cells, and produced a comparable increase in cells transfected with p800-luc. TGF-β1 treatment of the cells transfected with SmSmad4 sharply down regulated the luciferase activity in cells transfected with pCAL2 reporter, while it significantly increased the luciferase activity of the p800-luc-transfected cells (Fig. 7A). This demonstrates that SmSmad4 complements the human Smad4 deficiency and restores TGF-β responsiveness in these cells.

Mink lung epithelial cells, Mv1Lu, transfected with p800-luc reporter, were tested to further evaluate the activity of schistosome Smad proteins in heterologous cells. Cells transfected with the reporter construct alone showed a modest change in luciferase activity upon addition of TGF-β1. TGF-β1 treatment of cells transfected with either SmSmad2 or SmSmad4 alone exhibited a comparable change in luciferase activity. When Mv1Lu cells were transfected with both SmSmad2 and SmSmad4, the expression of luciferase reporter gene was elevated in the absence of TGF-β and further enhanced in response to TGF-β in a dose-dependent manner. The increase in luciferase activity significantly surpassed the effect of the

Smad plasmids transfected individually (Fig. 7B, left panel). A similar experiment was conducted on the Smad4-deficient cell line, MDA-MB-468. TGF-β1 treatment of cells transfected either with p800-luc reporter alone or co-transfected with SmSmad2 failed to boost the luciferase activity above the background level. Co-transfection with SmSmad4 significantly enhanced the luciferase activity in the absence or presence of TGF-β1. Cells co-transfected with both SmSmad4 and SmSmad2 exhibited a further increase in luciferase activity in the absence or presence of TGF-β1 (Fig. 7B, right panel).

**DISCUSSION**

The current investigation focuses on signaling pathways in the parasite Schistosoma, as an important step to identify the molecular mechanisms of biological events mediated by self or host signals. Such studies contribute to strategies aimed toward the development of therapies and vaccines for control of schistosomiasis. In higher organisms, TGF-β signaling controls a diverse set of cellular processes ranging from cell proliferation to apoptosis. Smad4 is a central mediator that plays an essential role in most TGF-β-mediated pathways. Smad4 exhibits the basic features characteristic of other co-Smads. The high degree of conservation in the MH1 and MH2 domains provided the means to identify the schistosome homologue. As is the case with other transcription factors, the intracellular distribution of Smad4 determines to a great extent its function.
The conservation of the NLS in the MH1 domain (38, 55) and NES in the N-terminal end of the linker region (38, 56) suggests that SmSmad4 is engaged in an active nucleocytoplasmic shuttling, a distinctive feature of co-Smads (57, 58). Smad4 activation domain or SAD is also well conserved in SmSmad4. SAD conservation is reflected in the retention of a proline-rich...
produced by a TGF-I receptor and a downstream R-Smad member(s), which synergize with SmSmad4 to orchestrate the biological events induced by a TGF-β-like ligand(s) in these stages of the parasite life cycle. This hypothesis is supported by the genomic analysis of the related nematode, Cuonarhabditis elegans, which revealed the presence of 4 TGF-β-like ligands that signal in two main pathways via two type-I receptors. These ligands initiate the signaling cascades by binding only one TGF-β type II receptor (Daf-4), which phosphorylates and activates both type-I receptors (Daf-1 and Sma-6). The later receptors in turn interact with and transduce the signal to 6 members of the Smad family of proteins (Sma-2, Sma-3, Sma-4, Daf-8, Daf-14, and Daf-3) (59).

SmSmad4 was found to interact with SmSmad1 and SmSmad2. Both in vivo and in vitro analyses demonstrate that SmSmad4 associates with SmSmad2 upon the phosphorylation and activation of the later by SmTβR-I. The inhibition of the interaction between SmSmad2 and SmSmad4 in the presence of wild-type receptor I could be attributed to the SmTβR-I binding of SmSmad2, thus preventing SmSmad2 binding to SmSmad4. Similarly, the interaction between the non-phosphorylatable forms of SmSmad2 with SmSmad4 is reduced in the presence of any form of receptor I. Our data and a previous report (60) show that SmSmad4 binds to the type I receptor, preferably to the constitutively active form. Further-

**Fig. 4.** Semi-quantitative RT-PCR analysis of SmSmad4, SmSmad2, and SmSmad1. RT-PCR analysis was performed on total RNA isolated from different developmental stages. Panel A represents a bar graph representation of the relative PCR band intensities (%) of SmSmad4, SmSmad2 and SmSmad1 as compared with that of Sm-α-tubulin. Values were calculated from three independent PCR amplifications (Error bars represent S.D.). Panel B shows the agarose gel electrophoresis of the PCR products of SmSmad4 (I), SmSmad2 (II), SmSmad1 (III), and Sm-α-tubulin (IV). Stages and the respective lane labels, shown in brackets, are: S. mansoni eggs (Eggs), S. mansoni in vitro transformed primary sporocysts (Sporocysts), 30-day infected B. glabrata (30d-inf. B.g.), and 45-day-old of the following worm populations: adult worm pairs (AWP), adult female worms (AWF), adult male worms (AWM), and female and male worms recovered from single sex infections (SSF and SSIM, respectively).

| AD construct/Ura3 | SmSmad4 | SmSmad2 | SmSmad2-3’ | SmSmad2-MH2 | SmSmad2-AAA | SmSmad1 |
|-------------------|---------|---------|-----------|-----------|-----------|---------|
| –                 | +/–     | +/–     | +         | +         | +         | ++      |
| SmSmad2           | +/–     | +/–     | –         | +         | ++        | –/–     |
| SmSmad2-3’        | +/–     | +/–     | –         | +         | ++        | –/–     |
| SmSmad2-MH2       | +       | +       | +/–       | +/–       | +/–       | +/–     |
| SmSmad2-AAA       | +       | +       | +/–       | +/–       | +/–       | +/–     |
| SmSmad1           | ++      | +       | ++        | +/–       | +/–       | ++/–    |

TABLE II

Yeast two-hybrid assay, in which SmSmad4 fused to GAL4 DNA-binding domain (SmSmad4-DBD) was co-transformed with different schistosome R-Smads constructs fused to GAL4 transcription activation domain (AD constructs). The presented data are collected from assays performed on AH109, YRG2, PJ69–4A, and Y187 yeast strains. Yeast three-hybrid assay, in which SmSmad4-DBD was co-transformed with any of the AD constructs of R-Smads and either wild type or the constitutively active version of receptor 1-Ura3 constructs, selected for with uracil removal (Ura3). Yeast host strains PJ69–4A and Y187 were used in the three-hybrid assay. The cumulative data presented in this table considered at least 2 of the following parameters: Number of colonies present on selective media as compared to control plates (number on control plates were between 200–500 colonies); the intensity and duration of development of the blue color in LacZ-filter assay; β-galactosidase units calculated from liquid LacZ assay using ONPG substrate. Negative sign (−) stands for undetectable interaction; (+/−), weak interactions (1–4 colonies grew on selective media, a faint blue-green color produced in LacZ-filter assay that requires incubation of about 3 days; and/or <0.5 β-galactosidase units in liquid LacZ assay; (+), positive interactions (5–10 colonies, greenish-blue color in LacZ-filter assay developed in 2–3 days, and/or 0.5–1.0 β-galactosidase units in liquid LacZ assay; (++) stronger positive interactions (11–25 colonies, blue color in LacZ-filter assay developed in 1–2 days and/or 1.0–2.0 β-galactosidase units in liquid LacZ assay).
FIG. 5. In vitro interaction of SmSmad4 with different schistosome R-Smads. A, co-precipitation of different SmSmads with SmTβR-I-wt (wt) or SmTβR-I-Q/D (Q/D). In vitro translated, unlabeled-S-tagged wt and Q/D were incubated with 35S-labeled, in vitro translation products of each of the following constructs: SmSmad2-MH2/AAA, SmSmad2-MH2, SmSmad4, SmSmad2 and SmSmad1. Binding reactions were purified using S-protein agarose beads, and the products were separated onto SDS-polyacrylamide gels (SDS-PAG) and subjected to autofluorography (panel I). The bar graph (panel II) shows the binding of each construct to each of SmTβR-I. Values are mean values obtained from two independent assays. B, GST pull-down analysis of SmSmad4 binding to wt and C-terminally tagged SmSmad2. GST (I) and GST fusion proteins of SmSmad2-linker (II), wild-type SmSmad2 (III), and C-terminally-tagged SmSmad2 (SmSmad2–3′/H11032) (IV) were expressed in bacteria, affinity-purified over glutathione-Sepharose beads and the protein-bound beads were allowed to interact with 35S-labeled, in vitro translated SmSmad4 in the presence or absence of in vitro translated SmTβR-I wt and Q/D. Binding reactions were separated by SDS-PAGE and subjected to autofluorography. Lanes are labeled according to the absence or the presence of receptor I. C, co-immunoprecipitation of SmSmad2-MH2 and SmSmad1 with SmSmad4. 35S-labeled, in vitro translated products of SmSmad2-MH2 (I), SmSmad2-MH2/AAA (II), and SmSmad1 (III) were immunoprecipitated using α-SmSmad4-linker IgG in the presence or absence of SmSmad4 and SmTβR-I (wt or Q/D). Immunoprecipitation products were separated by SDS-PAGE and subjected to autofluorography. Lanes are labeled to specify the input components of each reaction. In vitro translated products (20% of input in each reaction) are shown in the left lane of each panel. Bkg, background.
more, Beall and Pearce (60) reported that SmSmad1 was not phosphorylated by the Q/D form of receptor I nor could it be induced to stimulate luciferase activity in response to TGF-β1 for 4 h. Cells were transfected with control reporter plasmid pRL-CMV for normalizing data to transfection efficiency. Data are averages of triplicates from 2 independent assays, ± S.D. B, luciferase activity determined in mink lung epithelial cells (Mv1Lu) and MDA-MB-468 cells transfected with the luciferase reporter p800-luc alone or in the presence of SmSmad2, SmSmad4 or both. Cells were either left untreated (light gray) or treated with rhTGF-β1, 0.25 nm (dark gray) or 0.50 nm (black) for 4 h. The bar graphs measure the luminescence (RLU) of different cell samples, normalized to the readings of the control Renilla luciferase plasmid co-transfected in all samples. Data are averages of triplicates from two independent assays ± S.D.

was found to exert a substantial inhibitory effect on the interaction of SmSmad4 with the receptor-phosphorylated form of R-Smad (SmSmad2) but not on the non-activated form (SmSmad1 and SmSmad2), in vitro. In the human model, previous studies demonstrated that cells expressing an oncogenic hyperactive Ras mutant typically acquire loss of TGF-β antiproliferative responses. This effect is primarily attributed to the inhibition of the nuclear translocation of the Smad complex upon Erk-mediated phosphorylation of R-Smads, Smad1 (46) and Smad 2 and 3 (45, 47). An alternative mechanism of down-regulation of TGF-β signaling by oncogenic Ras was reported by Saha et al. (61) who showed that hyperactive Ras induced proteasome-dependent degradation of Smad4. Our results are consistent with the Ras-mediated regulation of TGF-β signaling as hypothesized by Saha et al. (61), as only SmSmad4 contains Erk-phosphorylation sites, while none of the currently identified schistosome R-Smads (SmSmad1, SmSmad2, and Smad8/9 homologue) contain Erk phosphorylation motifs. Since all Smad4 homologues, except the Drosophila Medea, contain at least one Erk-phosphorylation site, it is plausible that Erk-phosphorylation of Smad4 could induce the proteasome-mediated degradation and that such degradation is a secondary event to Erk phosphorylation.
Therefore, the broad spectrum of the inhibitory effects of the oncocogenic hyperactive Ras on TGF-β signaling could be due to a combined effect of Erk phosphorylation of both R-Smads and co-Smad and that the later could induce a proteasome-dependent proteolysis of co-Smad (61). However, this hypothesis awaits further investigation.

In MDA-MB-468 breast cancer cells, which are deficient in Smad4 (62), Smad4 deficiency accounts for the non-responsiveness to TGF-β (63). However, this also addresses unanswered question about the mechanism of co-Smad (61). However, this a combined effect of Erk phosphorylation of both R-Smads and co-Smad (62), Smad4 deficiency accounts for the non-responsiveness to TGF-β (63). However, this also addresses unanswered question about the mechanism of co-Smad (61). However, this also addresses unanswered question about the mechanism of co-Smad (61). However, this also addresses unanswered question about the mechanism of co-Smad (61).

Smad4-dependent proteolysis of co-Smad (61). However, this also addresses unanswered question about the mechanism of co-Smad (61). However, this also addresses unanswered question about the mechanism of co-Smad (61). However, this also addresses unanswered question about the mechanism of co-Smad (61).

Smad4 binds to the nucleus, the Smad complex binds to the promoter of target genes and regulates the expression of these genes in response to TGF-β. This demonstrates that Smad4 is able to function with the cellular Smad proteins, which indicates the integration of the TGF-β signaling network in this heterologous system.

When both Smad2 and Smad4 were co-expressed, a further enhancement of transcription was observed. Since Smad2 had no effect on its own, the further increase in activity is likely to be due to cooperation between the schistosome Smads in the host cells. Previous studies reported that Smad2 stimulated the luciferase activity of TGF-β-responsive luciferase reporter construct in response to TGF-β when overexpressed with either human TβRI (60) or with the activated form of schistosome receptor I, SmTβRI-Q/D (60) in R1B mink lung epithelial cells, a TβRI-deficient cell line. Smad2 was also shown to respond to human TGF-β1 induction by increasing its nuclear localization (10, 11), an additional evidence of integration of Smad2 in mammalian systems represented by mammalian cell lines. Herein, Smad4 demonstrates a synergism with Smad2 in response to human TGF-β induction, which indicates that Smad4 is functionally integrated in this system. The conservation of Smad4 functions in heterologous systems was previously demonstrated. In COS cells, Drosophila Medea was shown to interact with human Smad1 in the presence of activated BMP receptor (BMPR-II) and to interact with human Smad2 in the presence of activated TGF-β type I receptor (TβRI) (64). In Xenopus embryos, human Smad4 could act as a ventral mesoderm inducer, mimicking the effect of low activin concentrations (24).

The identification of Smad4 raises many questions to be addressed such as the possibility of another receptor-I and an associated R-Smad, which might work upstream of Smad4 in the snail host. Another issue is the identification of the ligands, whether host or self in origin, and the different pathways mediated by Smad1, Smad2, or other yet to be identified R-Smad. TGF-β signaling pathways cross-talk via Smad4, which participates in heteromeric Smad complexes with different R-Smads and then the newly formed complex moves into the nucleus where it modulates target genes transcription. After being imported to the nucleus, the Smad complex binds to a nuclear partner, which directs the complex to the target gene(s) in order to elicit the specific effects/phenotypes that fit the tissue and/or the developmental stages. These downstream nuclear partner(s), and the target gene(s) are more issues that await answers.

On the other hand, Smad4 identification has also addressed unanswered questions about the mechanism of interaction of schistosome R-Smads with co-Smad in response to an activating signal and how the phosphorylation status of the Smad protein regulates its interaction and consequently its function. In conclusion, the identification of Smad4 homologue in S. mansoni, a central TGF-β signal transducer, is an important step toward the characterization of the TGF-β pathways in schistosomes.
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Expression of Functional Schistosoma mansoni Smad4: ROLE IN ERK-MEDIATED TRANSFORMING GROWTH FACTOR β (TGF-β) DOWN-REGULATION
Ahmed Osman, Edward G. Niles and Philip T. LoVerde

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