Identification of STRAP, a Novel WD Domain Protein in Transforming Growth Factor-β Signaling*

(Received for publication, September 3, 1998, and in revised form, October 28, 1998)
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Transforming growth factor-β (TGF-β) is the prototype of a large family of proteins that regulate a variety of biological processes. The pleiotropic responses to TGF-β are mediated via ligand-induced heteromeric complex formation by type I (TβR-I) and type II (TβR-II) serine-threonine kinase receptors. Several studies have shown that TβR-II acts as a primary receptor, binding TGF-β and phosphorylating TβR-I, whose kinase activity then propagates the signals. Therefore, intracellular proteins that interact with type I receptors are likely to play important roles in TGF-β signaling. We have identified a novel WD domain-containing protein, designated STRAP (serine-threonine kinase receptor-associated protein), which interacts with TβR-I in a yeast two-hybrid system. STRAP associates with both functional TβR-I and TβR-II in vivo. Overexpression of STRAP leads to inhibition of TGF-β-mediated transcriptional activation. It also shows synergistic inhibition of TGF-β signaling in concert with Smad7, but not with Smad6, as measured by TGF-β-dependent transcriptional reporters. The existence of the STRAP gene from yeast to mammals indicates an evolutionarily conserved function in eukaryotes. The data suggest a potential role for STRAP in TGF-β signal transduction.

TGF-β1 exert their wide range of biological effects such as cell proliferation, differentiation, matrix production, and apoptosis (1, 2) through binding to specific cell surface receptors. Upon ligand binding, the type II receptor, which is a constitutively active kinase, transphosphorylates the type I receptor at serine and threonine residues clustered in the GS domain (3). Previous studies suggest that TβR-II, acting downstream of TβR-II, is directly involved in transducing TGF-β signals to downstream effectors, including SMAD proteins (3, 4).

SMAD proteins are classified according to their role in signaling by TGF-β family members. Pathway-restricted SMADs interact transiently with and are phosphorylated by specific activated type I receptors. Smad2 and Smad3 mediate signaling by TGF-β and activin, whereas Smad1 and Smad5 are involved in BMP signaling. Smad4 is a common mediator of TGF-β, activin and BMP signals (3, 4). Smad6 and Smad7 function as inhibitors of these signaling pathways by interfering with the activation of pathway-restricted SMADs (5–8). Other pathways may be important for the transduction of specific signals. Using genetic complementation in yeast, a serine-threonine kinase of the mitogen-activated protein kinase kinase kinase family, TAK1, was identified as an alternative mediator of TGF-β signals (9).

Using yeast two-hybrid screens, several candidates interacting with TβR-I or TβR-II have been identified that may modulate receptor signaling. The FK506 and rapamycin-binding immunophilin, FKBP12, interacts with unstimulated TβR-I and other type I receptors. FKBP12 is not a substrate for the receptor kinase but inhibits its signaling function (10, 11). The α-subunit of farnesyltransferase can interact with TβR-I, and although it is phosphorylated by TβR-I (12, 13), little is known about its role in TGF-β signaling. The C-terminal portion of TRAP-1 is sufficient for interaction with activated TβR-I and was shown to inhibit TGF-β signaling (14). Using a similar approach, TRIP-1 was identified as a TβR-II interacting protein, whose functional importance is not known (15).

Although the nature and mechanism of activation of TGF-β receptors at the cell surface has been described and the roster of potential regulators of TGF-β signaling continues to expand, little is known at the molecular level about the signaling mechanisms immediately downstream of TGF-β receptors. To search for intracellular signal mediators that interact with the cytoplasmic region of TβR-I, we utilized a yeast two-hybrid system. One of the identified clones encoded a novel, WD domain-containing protein (16), STRAP, which interacts with both type I and type II TGF-β receptors in vivo. Moreover, we show that this gene is evolutionarily conserved and can negatively regulate gene expression from TGF-β-responsive promoters.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Assays and Interaction Cloning of STRAP—A mouse embryonic cDNA expression library in yeast Y190 was used for two-hybrid screening as described (17, 18). To serve as bait, the cytoplasmic domain of R4 (R4) (R4C amino acids 146–501) was fused in-frame to the 3’ end of the GAL4 DNA-binding domain in pA22. The resulting clones were subjected to yeast mating assay for interaction specificity using the Y187 strain of the opposite mating type containing the unrelated proteins p53, lamin, SNF1, and CDK2 fused to the GAL4 DNA-binding domain and were tested for β-galactosidase activity. Clones specific for R4C were further tested for specific interaction. STRAP cDNA was used as a probe to screen a mouse embryonic (8.5–9.0 days postcoitus) cDNA library in αZAP II (Stratagene) according to the manufacturer’s protocol. The inserts of five clones of thirteen positive clones showed an in-frame stop codon (TGA) 72 base pairs upstream from the ATG start codon and Kozak consensus sequence (20). The GenBank™ accession number for STRAP is AF096285.

Northern Blot and Southern Blot Analysis—A blot (CLONTECH) containing mRNA (2 μg/lane) from multiple mouse tissues was prehybridized and hybridized according to the manufacturer’s instructions. The blot was probed with 32P-labeled STRAP and actin cDNAs sequentially. Similarly, a membrane (CLONTECH) containing genomic DNA (4 μg/lane) from various eukaryotic species, digested with EcoRI, was
probed with 32P-labeled STRAP.

In Vivo Interaction Assay—The complete coding region of STRAP was amplified by polymerase chain reaction and subcloned into a mammalian expression vector, pcDNA3 (Invitrogen), with one copy of the Flag epitope in-frame to the C terminus to generate STRAP-Flag. Similarly STRAP-HA was made. All receptor constructs were kindly provided by Dr. Joan Massagué. COS-1 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Cells were transiently transfected with indicated plasmids using a calcium-phosphate precipitation method (21). After 40 h, cells were washed and fed Eagle’s medium supplemented with 10% fetal bovine serum. Cells were subjected to immunoprecipitation with anti-Flag M2 monoclonal antibody (IBI, Eastman Kodak), anti-TβR-I polyclonal antibody (Santa Cruz), or anti-TβR-II polyclonal antibody (Santa Cruz) followed by adsorption to protein G-Sepharose (Amersham Pharmacia Biotech). The beads were washed four times with lysis buffer. The immune complexes were analyzed by SDS-PAGE, and tagged proteins were immunoblotted with either anti-Flag antibody or anti-HA polyclonal antibody (Y11, Santa Cruz) and detected using an enhanced chemiluminescence system.

Affinity Cross-linking—Transfected COS-1 cells were affinity labeled using 125I-TGF-β1 by chemical cross-linking as described (22). Cell lysates were subjected to immunoprecipitation with anti-Flag antibody, and affinity labeled receptor complexes coprecipitated with STRAP were visualized by SDS-PAGE and autoradiography.

Transcriptional Response Assay—Mv1Lu or its derivative R1B/L17 cells were seeded at 40–60% confluence in 12-well plates and transfected 24 h after plating using a DEAE-dextran transfection method (Promega). After overnight recovery cells were incubated in Dulbecco’s modified Eagle’s medium + 0.2% fetal bovine serum with or without 100 pM TGF-β1 for 20 h. Luciferase activity and β-galactosidase activity were measured in an Analytical Luminescence Labs Monolight 2010 Luminometer.

RESULTS

Using the cytoplasmic domain of type I TGF-β receptor from rat (R4C) as the bait, we performed a yeast two-hybrid screen (17,18) of a mouse embryonic cDNA library. Fourteen positive clones that specifically interacted with R4C were obtained from ~6 × 10^6transformants and subdivided into three groups based on sequence analysis. Twelve clones encoded FKBP12, which was previously shown to interact with several unrelated proteins including lamin, SNF1, p53, or CDK2 in mammals (Fig. 2A). The STRAP clone yielded a 1648-base pair cDNA with an apparent molecular mass of 39 kDa on SDS-PAGE (data not shown). Sequence analysis indicates that STRAP contains six WD domains. A search of GenBank™ using BLAST and the alignment program MegAlign revealed maximum similarity (98%) in the amino acid level with another WD repeat protein FKBP12.6, which interacted strongly. Some of these similarities are among the conserved amino acid residues within the WD repeats.

To analyze the expression pattern of the STRAP gene, Northern blot analysis of poly(A)+ RNA from different mouse tissues was performed (Fig. 2A). A major transcript of 1.8 kb was detected in all tissues examined, with the highest levels in liver and testis and lesser abundance in spleen. In some tissues, larger transcripts were detected. Northern blot analysis of poly(A)+ RNA from different cell lines including Mv1Lu, 293R, R1B/L17, COS-1, and MCF7 showed one message of 2.0 kb (data not shown). Southern blot analysis of genomic DNA from different species was performed using the STRAP cDNA as a probe. The presence of different size fragments in all species tested indicated conservation of the STRAP gene from yeast to mammals (Fig. 2B).

To investigate the association of STRAP with TβR-I in vivo, STRAP-Flag was transfected into COS-1 cells alone or in combination with HA-tagged wild type and several mutant forms of TβR-I. Cell lysates were subjected to immunoprecipitation with antibodies to Flag, and each immunoprecipitate was then probed with antibodies to HA. A comparable amount of TβR-I was detected in each immunoprecipitate (Fig. 3A, first panel, lanes 4–7), revealing that TβR-I can be communoprecipitated
with STRAP. Reciprocal experiments in which proteins immunoprecipitated by antibodies to TβR-I were blotted with an anti-Flag antibody (Fig. 3A, second panel) confirmed the association of STRAP with ligand-free TβR-I. This association was minimally affected by the mutations K232R (kinase negative), T204D (constitutively active), or 185–204 (activation incompetent) in TβR-I (24). To determine whether STRAP can also interact with TβR-II, similar experiments were performed using HA-tagged TβR-II coexpressed with STRAP-Flag (Fig. 3B). TβR-II was coimmunoprecipitated with STRAP (top panel) and vice versa (bottom panel), demonstrating that STRAP interacts with TβR-II in vivo. But STRAP did not interact under similar conditions with the unrelated proteins RhoB or Smad1 when coexpressed in COS-1 cells (data not shown). HA-tagged STRAP also associated with Flag-tagged receptors, demonstrating that the association was independent of the epitope tag employed. Additionally, coimmunoprecipitation of both receptors with STRAP (lane 3) was not affected by the treatment of cells with TGF-β1 (Fig. 3C, lane 4). Finally, we examined the association of STRAP with the TGF-β receptor complex in affinity cross-linking experiments. 125I-TGF-β1-bound TβR-I-TβR-II heteromeric complexes could be coimmunoprecipitated with STRAP (Fig. 3D). Thus, STRAP associates specifically with TβR-I and TβR-II in mammalian cells.

To explore the functional significance of the interaction between STRAP and TGF-β receptors we focused our analyses on a TGF-β-responsive reporter, p3TP-Lux, which contains elements from the PAI-1 promoter and drives expression of a luciferase reporter gene (24). TβR-I restores responsiveness to TGF-β in transfected RII/L17 cells (Fig. 4A). Overexpression of STRAP suppressed the TGF-β-induced increase in luciferase activity in a dose-dependent manner. Induction of p3TP-Lux by TGF-β was decreased from 51- to 23-fold by 1 μg of STRAP expression plasmid. Analogous inhibition was observed in HepG2 cells using another TGF-β-responsive reporter (CAGA)9 MLP-Luc, which contains multiple Smad3/Smad4 binding CAGA boxes upstream of a minimal adenovirus major late promoter (data not shown) (25). To examine whether STRAP can cooperate with inhibitory SMADs in the inhibition of TGF-β-induced transcription, Mv1Lu cells were transfected with the reporter p3TP-Lux and expression plasmids as shown in Fig. 4B. Smad7 inhibited TGF-β-induced luciferase activity as expected (7, 8). Coexpression of Smad7 with STRAP resulted in synergistic inhibition of p3TP-Lux transactivation in response to TGF-β. However, STRAP did not synergize with Smad6, an antagonist of BMP signaling (6). Similar results were observed with the (CAGA)9 MLP-Luc reporter. These results suggest that STRAP can function as a negative regulator of transcription by TGF-β.

DISCUSSION

Members of the TGF-β family exert a wide range of biological effects on a large variety of cell types. Numerous studies have been conducted to understand the signal transduction pathways downstream of the TGF-β receptor complex. Recent evidence suggests that SMAD proteins are clearly crucial for...
STRAP in TGF-β Signaling

Figure 4. TGF-β-induced transcriptional activation of the 3TP promoter is inhibited by STRAP. A, R1B/L17 cells were transiently transfected with β-galactosidase reporter, β-galactosidase reporter, TβR-I (0.3 μg), and increasing amounts of STRAP (0.2, 0.5 and 1 μg). In each experiment total equal amounts of DNA were transfected. Cells were subsequently incubated in the absence or presence of 100 pm TGF-β1 for 20 h, and relative luciferase activity was measured in cell lysates. Luciferase activity was normalized to β-galactosidase activity. Data are the means ± S.E. of triplicate determinations from a representative experiment. B, STRAP synergizes with Smad7 to inhibit TβR-I(204D)-induced 3TP promoter activity in Mv1Lu cells. Cells were transfected with Smad7 (0.3 μg), Smad6 (0.52 μg), and increasing amounts of STRAP (0.2, 0.5 and 1 μg) as indicated. Transfections and luciferase assays were carried out as described above. These experiments were performed in triplicate four times with similar results.

signal transduction by members of the TGF-β family (3, 4). However, it is likely that other signaling pathways may exist for transducing specific signals. The SMAD signaling pathways, which offer combinatorial diversity, can be modulated at multiple levels. At the receptor level, the immunophilin FKBP12 binds to TβR-I and inhibits its signaling function (10, 11). Other receptor-binding proteins such as TRAP-1 (14), far-neryltransferase-α (12, 13), and TRIP-1 (15) similarly might have regulatory roles. In our study, the findings that STRAP associates with both types of TGF-β receptors and inhibits TGF-β-induced transcription in concert with its synergistic inhibition with Smad7 suggest that STRAP is a potential candidate in mediating TGF-β signals. We also report here the interaction between TβR-I and FKBP12.6, a member of the FKBP family (23). This interaction may also have some inhibitory effect in TGF-β signaling, like FKBP12.

Sequence analysis of the predicted open reading frame encoding STRAP revealed it to be a member of the family of WD repeat proteins (16). WD repeat proteins appear to serve regulatory functions in various cellular processes. Many WD repeat proteins are involved in signal transduction, such as the β-subunit of heterotrimeric G proteins, RACK1, PLAP, the regulatory subunit of phosphatase 2A, or TRIP-1 (26). Many WD repeat proteins help to assemble macromolecular complexes, as shown for the β-subunit of G proteins (27). Such proteins present a changeable surface for protein-protein interaction and are capable of protein-induced conformational changes. STRAP associates with both receptors, and it can form homo-oligomers (data not shown) most likely through WD repeats. STRAP showed synergistic inhibition of TGF-β signaling with Smad7, as measured by the βmTP-Lux and (CAGA)₅ MLP-Luc reporters. Smad7 interacts with the TGF-β type I receptor in the presence of TGF-β, thereby blocking the association, phosphorylation, and activation of Smad2 and Smad3, thus inhibiting TGF-β signaling (7, 8). It is possible that STRAP stabilizes the complex between Smad7 and the receptors to inhibit TGF-β-dependent gene transcription. In contrast, STRAP did not synergize with Smad6. This is consistent with both a distinct mechanism of inhibition for Smad6 and its primary role in regulating BMP signals (6). Analogous to the recruitment of signaling components to receptor tyrosine kinases, STRAP may be involved generally in recruiting downstream regulatory molecules to the receptor serine-threonine kinases. Future studies will investigate the involvement of STRAP in TGF-β signaling via SMADs or other pathways.

Acknowledgments—We thank S. Elledge, J. Massagué, E. Olson, M. Kawabata, X.-F. Wang, P. Donahoe, P. ten Dijke, J.-M. Gauthier, and R. Barstead for the generous gifts of two-hybrid screen reagents, strains, plasmids, and cDNA libraries. We are grateful to M. Engel for critical reading the manuscript.

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J. Biol. Chem. 1998, 273:34671-34674.
doi: 10.1074/jbc.273.52.34671

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