A Novel Protein Distinguishes between Quiescent and Activated Forms of the Type I Transforming Growth Factor β Receptor*

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Transforming growth factor β (TGFβ) signal transduction is mediated by two receptor Ser/Thr kinases acting in series, type II TGFβ receptor (TβR-II) phosphorylating type I TGFβ receptor (TβR-I). Because the failure of interaction cloning, thus far, to identify bona fide TβR-I substrates might reasonably have been due to the use of inactive TβR-I as bait, we sought to identify molecules that interact specifically with active TβR-I, employing the triple mutation L193A,P194A,T204D in a yeast two-hybrid system. The Leu-Pro substrates prevented interaction with FK506-binding protein 12 (FKBP12), whose putative function in TGFβ signaling we have previously disproved; the charge substitution at Thr204 constitutively activates TβR-I. Unlike previous screens using wild-type TβR-I, where FKBP12 predominated, none of the resulting colonies encoded FKBP12. A novel protein was identified, TβR-I-associated protein-1 (TRAP-1), that interacts in yeast specifically with mutationally activated TβR-I, but not wild-type TβR-I, TβR-II, or irrelevant proteins. In mammalian cells, TRAP-1 was co-precipitated only by mutationally activated TβR-I and ligand-activated TβR-I, but not wild-type TβR-I in the absence of TGFβ. The partial TRAP-1 protein that specifically binds these mutationally and ligand-activated forms of TβR-I can inhibit signaling by the native receptor after stimulation with TGFβ or by the constitutively activated receptor mutation, as measured by a TGFβ-dependent reporter gene. Thus, TRAP-1 can distinguish activated forms of the receptor from wild-type receptor in the absence of TGFβ and may potentially have a functional role in TGFβ signaling.

The signal transduction events coupling receptors for the TGFβ superfamily to TGFβ-dependent responses remain incompletely understood. TGFβ signaling requires two transmembrane receptors acting in series, TβR-II phosphorylating TβR-I, each characterized by a cytoplasmic serine/threonine kinase domain (1), but the subsequent signaling pathways are less clear-cut (2). Several candidates have been identified by interaction cloning in yeast “two-hybrid” systems, which interact with the cytoplasmic domain of TβR-I. These include both the immunophilin-binding protein, FKBP12 (3), a target for the macrolides FK506 and rapamycin, and the α subunit of Ras farnesyltransferase (FNTA) (4, 5). TβR-II-interacting protein 1, a Trp-Asp domain protein, was isolated analogously (6). FKBP12 associates with inactive ALK5 and is released from receptor complexes upon ligand binding (3). Although FKBP12 might inhibit TGFβ signaling, at least at threshold concentrations of ligand (7, 8), mutational analysis by ourselves (9) and others (8, 10) has proven that FKBP12 recognition is dispensable for signal generation by TβR-I; FNTA likewise is unnecessary for TGFβ signaling (11). However, genetic screening in Drosophila identified the transcription factor, mothers against decapentaplegic (MAD), as acting downstream from the TGFβ homolog, decapentaplegic (12). In vertebrates, multiple MAD-related proteins exist (Smads) that are thought to mediate signaling by TGFβ family members via phosphorylation-dependent nuclear translocation (13–17). TGFβ responses required Smad4 in concert with Smad2 (14) or Smad3 (14, 15). Because Smad proteins are substrates for type I receptors (16, 17), these transcription factors may provide a direct link between type I receptors and the nucleus. Using genetic complementation in yeast, a novel member of the mitogen-activated protein kinase family, the TGFβ-activated kinase, TAK1, was identified as an alternative mediator of TGFβ signaling, which may be necessary for at least a subset of TGFβ effects (18). Thus, functional pathways appear to exist distinct from direct phosphorylation of Smad proteins.

Because the failure of interaction cloning to identify bona fide TGFβ receptor substrates might reasonably have been due, in part, to the use of inactive receptor as bait, we sought to identify molecules that interact specifically with active TβR-I, employing TβR-I.L193A,P194A,T204D as the bait. The charged amino acid substitution at Thr204 confers constitutive activity in the absence of ligand and TβR-II; disruption of the invariant Leu-Pro motif abrogates binding to FKBP12 (9). Using this strategy we identified a novel protein, TRAP-1, that discriminates between quiescent TβR-I and TβR-I that is activated in the presence of TGFβ.

EXPERIMENTAL PROCEDURES

Interaction Cloning and Two-Hybrid Assays in Yeast—Constructions containing the cytoplasmic domains of TβR-I and TβR-II or point mutations of TβR-I in the yeast expression plasmid pAS2–1 were previously described (9). The cytoplasmic domain of TβR-I.L193A,P194A,T204D in pAS2–1 was used as bait, to screen a human lymphocyte cDNA library in the pACT vector (CLONTECH). The bait and library DNA were

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† The abbreviations used are: TGFβ, transforming growth factor β; FKBP12, FK506-binding protein 12; FNTA, α subunit of farnesyltransferase; RACE, rapid amplification of cDNA ends; SD, synthetic dropout; Smad, MAD-related protein; TβR-I, type I TGFβ receptor; TβR-II, type II TGFβ receptor; TRAP-1, TβR-I-associated protein-1; 3-AT, 3-amino-1,2,4-triazole; bp, base pair(s); DMEM, Dulbecco’s modified Eagle’s medium; HA, hemagglutinin.
Introduction of TRAP-1 cDNA into yeast strain Y190 (GAL1-URAS1-GAL1-TATA-HIS3 and GAL1-URAS1-GAL1-TATA-lacZ) using lithium acetate (19). Transformants were selected on synthetic dropout (SD)/-Leu/-Trp/-His+/-25 μm 3-amino-1,2,4-triazole (3-AT) plates and tested for lacZ using a β-galactosidase assay (20). A lift assay (9) was performed on SD/-Leu/-Trp/-His+/-25 μm 3-AT and IAHG adenine plates to eliminate the bait plasmid and then subjected to the yeast mating assay for interaction specificity. As an additional control for the two-hybrid assays in yeast, VP16-3, encoding murine p53 (amino acids 72–390), and pLAM5 (amino acids 66–230), were obtained from CLONTECH. Candidate plasmids purified from yeast were sequenced by the dideoxy method.

To obtain a more complete TRAP-1 cDNA, the 3′ partial cDNA was used to screen a human heart cDNA library in Agt10 (CLONTECH); 50,000 plaques were plated per 150-mm dish. Duplicate filters were hybridized with 32P-labeled TRAP-1 cDNA (Rediprime DNA labeling kit, Amersham Pharmacia Biotech) in 7% SDS, 0.5 M NaH2PO4, and 1 mM EDTA at 55 °C overnight. Filters were washed twice in 2× SSC and 0.05% SDS at room temperature for 15 min and then in 0.1× SSC and 0.1% SDS twice at 55 °C for 15 min. Filters were subjected to autoradiography. Purification of positive clones was done as described (20). Inserts were subcloned into pBlueScript SK (Stratagene) and sequenced. The GenBank accession number for TRAP-1 is AF022795.

5′-Rapid Amplification of cDNA Ends (RACE)—The 5′ sequence of TRAP-1 was completed using the 5′-RACE reaction. Human heart pRNA (CLONTECH) was reverse transcribed using the TRAP primer-1, 100 ng of the pCH110 TGF-β receptor-associated protein (encoding the C-terminal 387 amino acids) was excised from pAS2–1 TRAP-1–CMV2, the partial TRAP-1 cDNA expression plasmid pFlag-DRepressor-1, 100 ng of the pCH110 β-galactosidase reporter gene, driven by the SV40 early promoter (Amersham Pharmacia Biotech) (25), 0–600 ng of pFlag-ΔTRAP-1-CMV2, and 0–100 ng of a CMV-driven β-galactosidase plasmid (26) in cotransfection with 50 μg of wild-type or mutant TGF-β receptor-1 (R-I, HaloTag) and TGF-β1 (10 ng/ml R & D Systems) or other test peptide. Dipeptide. Five clones corresponded to FNTA, which previously had been shown to specifically with TGF-β1, T204D but not with wild-type TGF-β1, R-I as Bait—A human lymphocyte cDNA library was screened using TGF-β1-1H10, P194A, T204D as bait in the yeast two-hybrid system (24). Mammalian 293 cells, encoding both chimeric proteins into a single yeast host (16). Yeast strain Y187 (MATα) was transformed with wild-type Tgf-β1, point mutations of Tgf-β1, or Tgf-β2 plasmids and grown on SD/-Trp plates. Yeast strain Y190 (MATα) was transformed with TRAP-1 or TRAP-2 and grown on SD/-Leu plates. One colony from each mating type was picked, placed in 0.5 ml of YPD medium (20 g/liter peptone, 10 g/liter yeast extract), and incubated at 30 °C with shaking at 250 rpm for 2 h. Matinal mating TRAP-1 cDNA were spread on SD/-Leu/-Trp and SD/-Leu/-Trp/-His+/-25 μm 3-AT plates and incubated at 30 °C for 5 days. Growth was scored on SD/-Leu/-Trp/-His+/-25 μm plates. The β-galactosidase colony lift filter assay was performed on SD/-Leu/-Trp plates (22).

Northern Blot Analysis—Filters containing poly(A)+ RNA from multiple human tissues (CLONTECH) were prehybridized in ExpressHyb solution (CLONTECH) at 68 °C for 30 min and hybridized with 32P-labeled TRAP-1 cDNA probe at 68 °C for 1 h. Filters were washed in 2× SSC and 0.05% SDS at room temperature for 30 min, washed in 0.1x SSC and 0.1% SDS at 50 °C for 40 min, and subjected to autoradiography.

Co-precipitation in Mammalian Cells—To engineer the mammalian expression plasmid pFlag-ΔTRAP-1-CMV2, the partial TRAP-1 cDNA (encoding the C-terminal 387 amino acids) was excised from pAS2–1 and inserted into pFlag-CMV-2 (IBI Kodak). Mammalian 293 cells, plated at a density of 5×104 cells/cm2 in 100-mm tissue culture dishes, were cotransfected with 5 μg of wild-type or mutant Tgf-β1-HA-pCMV5, 8 μg of pFlag-ΔTRAP-1-CMV2, 8 μg of Tgf-β1-II-SV-Sport, and 45 μl of LipofectAMINE (Life Technologies, Inc.). Human recombinant Tgf-β1 (1 ng/ml R & D Systems) was added to the culture medium without or with 100 ng/ml antibody (Clontech) to neutralize the TGF-β1 at 30 °C with shaking at 250 rpm for 24 h. Mammalian cells were harvested by centrifugation, lysed in lysis buffer, and subjected to Western blot analysis. The proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and were transferred electrophoretically to nitrocellulose membranes. The membranes were incubated for 1 h at room temperature with 1 μg/ml anti-HA-biotin (Boehringer Mannheim), washed 4× with Tris-buffered saline:0.05% Tween, and incubated with 0.2 units/ml streptavidin-peroxidase (Boehringer Mannheim). Bound antibody was detected using the enhanced chemiluminescence system (Amersham Pharmacia Biotech).

Transcriptional Control—HepG2 hepatocellular carcinoma cells (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 20 mM HEPES, pH 7.4, 6 mM NaHCO3, 10% fetal bovine serum, 1% nonessential amino acids, 1% glucose, and 1% sodium pyruvate. Cells were transfected 24 h after plating by a calcium phosphate method (23), using 100 ng of pTGF-β1-1H10, P194A, T204D for 1 h and incubated 24 h with 50 μg/ml of DMEM with 10% fetal bovine serum 1 h before transfection. Calcium-phosphate DNA precipitates were formed by slowly mixing 20 μl of 50 mM HEPES, pH 7.1, 280 mM NaCl, Na2HPO4, pH 7.0, 20 μl of 125 mM CaCl2 containing 0.8 μg of DNA. Cells were incubated with DNA precipitates (40 μg) for 5 h and were cultured overnight in DMEM with 10% fetal bovine serum. Medium was replaced on the following day by DMEM with 0.03% fetal bovine serum in the absence or presence of 1 ng/ml TGF-β1 (R & D Systems). Cells were harvested 24 h later, and the luciferase activity and β-galactosidase activity were measured. For all comparisons, total DNA and promoter content were kept constant using equivalent amounts of vector. Luciferase activity was corrected for the internal constitutive lacZ plasmid, and results (mean ± S.E.) are expressed relative to expression in vehicle-treated, vector-transfected cells (six to nine cultures for each condition, from two to three independent experiments). Results were compared by analysis of variance and Scheffe's test using a significance level of p < 0.01.

RESULTS

Interaction Cloning of a Novel Protein, TRAP-1, Using Activated Tgf-β1 as Bait—A human lymphocyte cDNA library was screened using Tgf-β1-L1H10, P194A, T204D as bait in the yeast two-hybrid system (24). Unlike previously reported screens using wild-type Tgf-β1, where FKBP12 was the predominant protein detected, none of the resulting colonies encoded FKBP12, as expected from the obligatory role of the Leu-Pro dipeptide. Five clones corresponded to FNTA, which previously was shown to interact with both Tgf-β1-I and T204D-Tgf-β1-I (4). Two proteins were novel, designated Tgf-β1-associated protein-1 and -2 (TRAP-1 and TRAP-2, Fig. 1). To define the binding specificity of TRAP-1 and TRAP-2, we employed the yeast mating assay to ensure co-introduction of plasmids encoding both chimeric proteins into a single yeast host (22). Yeast strain Y187 cells containing pFlag-ΔTRAP-1-CMV2-TRAP-2 plasmids were mated with yeast Y187 cells containing the “bait” (GALA-AD-Tgf-β1-L1H10, P194A, T204D) or other test cDNAs, in frame with the GAL4 activator domain. In this assay system, interaction between two hybrid proteins induces two GAL4-dependent genes, HIS3 and lacZ, which allows both growth on histidine-deficient plates and induction of β-galactosidase. By both criteria, TRAP-1 and TRAP-2 interacted specifically with Tgf-β1-L1H10, P194A, T204D but not with wild-type.
Fig. 2. TRAP-1 associates selectively with activating mutations of TβR-I. The specificity for interaction of TRAP-1 and TRAP-2 with TβR-I, mutations of TβR-I, and control proteins was assessed by the yeast mating assay. Yeast strain Y190 was transformed with TRAP-1 or TRAP-2 in pAS2-1 and was mated with yeast strain Y187 carrying wild-type or mutant TβR-I, TβR-II, lamin C, or p53 in pACT2. In A and B, mating cultures were plated on nonselective medium (SD/-Leu/-Trp, a and d) to confirm the presence of both plasmids and on selective medium (SD/-Leu/-Trp/-His/-1-3-AT; b and c) to assess protein-protein interaction by two-hybrid induction of the HIS3 gene; lacZ activity was determined by a colony lift X-gal assay on filter replicas of the SD/-Leu/-Trp plates (a and b).

Fig. 3. Northern analysis of TRAP-1 mRNA. Blots containing poly(A)+ RNA from multiple human tissues were hybridized with the radiolabeled 3’-1399-bp TRAP-1 cDNA. Two transcripts of 4.4 and 6 kilobases were detected in all tissues examined.

TRAP-1 Selectively Binds Active Forms of TβR-I in Yeast Two-hybrid Assays—Because three amino acid substitutions were incorporated in our TβR-I bait, to establish which amino acid(s) conferred this interaction, the associations between TRAP-1 and TRAP-2 and each individual mutation of TβR-I were tested. TRAP-1 also bound the TβR-I/T204D kinase domain, containing the substitution that activates TβR-I signaling; TRAP-1 did not interact with TβR-I/L193A or TβR-I/T204A (Fig. 2B). Thus, activation of TβR-I, not disruption of FKBP12 binding, was responsible for the conditional binding of TRAP-1. In contrast, TRAP-2 only interacted with TβR-I/L193A,P194A,T204D but not TβR-I/T204D or other single mutations. For this reason, TRAP-1 was selected for more extensive analysis.

To obtain the full-length coding sequence of TRAP-1, the 3’-1399-bp partial cDNA was used to screen a human heart cDNA library, and the 5’ sequence of TRAP-1 was obtained by performing 5’-RACE on poly(A)+ RNA from human heart. The full-length cDNA was 3100 bp in length, comprising 860 amino acid residues (Fig. 1). An in-frame stop codon (TAA) was 153 bp upstream from the ATG start codon and Kozak consensus sequence (26). A search of GenBank® using BLAST revealed no homologous proteins; a search for potential functional motifs using the BLAST Enhanced Alignment Utility identified one potential phosphorylation site for protein kinase A, nine for protein kinase C, and eight for casein kinase 2, and seven potential myristylation sites. No other known structural motifs were identified. TRAP-1 mRNA was detected as two transcripts of 4.4 and 6 kilobases in all tissues examined, with lesser abundance in lung and liver (Fig. 3); the mechanism for generating the two transcripts remains to be determined.

Fig. 4. TRAP-1 specifically binds ligand-activated TβR-I. Human 293 cells transfected with epitope-tagged TβR-I and ΔTRAP-1 constructs were incubated with vehicle versus TGFβ1. Cells were lysed, antibody to the FLAG epitope was added to immunoprecipitate (IP) TRAP-1 and associated proteins; immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis, and TRAP-1-associated TβR-I was detected with anti-HA antibody. Wt, wild type.

Fig. 5. A receptor-binding fragment of TRAP-1 inhibits TGFβ signaling. HepG2 cells were transfected with pβTP-Lux, the constitutive lacZ gene, and increasing amounts of the ΔTRAP-1 expression vector, pβTP-Lux expression was triggered using the constitutively activated receptor, TβR-I/L193A,P194A,T204D (A) or 1 ng/ml TGFβ1 without exogenous receptor (B). Results in A and B are shown relative to expression in vector-transfected and vehicle-treated cultures (n = 9 and 6, respectively). * and **, p = 0.0001 and p = 0.0002, in comparison with the absence of TRAP-1.
and then were blotted with antibody to the HA tag (Fig. 4). The constitutively activated receptor, TβR-I 



P194A.A204D, was coprecipitated with TRAP-1 even in the absence of TGFβ1. However, wild-type TβR-I-IA was coprecipitated only from TGFβ1-treated cells. This preferential binding to the ligand-activated receptor contrasts markedly with FKBP12 and far-nesyltransferase, both of which were released from TβR-I in the presence of TGFβ1 (5). Thus, unlike these proteins, TRAP-1 is recruited selectively to the active forms of TβR-I.

TRAP-1 Can Inhibit TGFβ-dependent Transcription—To ascertain whether binding of TRAP-1 to the activated receptor might have functional consequences, HepG2 cells were transfected with the TGFβ-responsive p3TP-Lux reporter gene, an internal lacZ control, and 0–600 ng of the partial TRAP-1 expression vector (Fig. 5). TβR-I 



P194A.A204D was sufficient to up-regulate expression more than 30-fold (33.5 ± 3.4; p = 0.0001 versus vector-transfected cells). Co-transfection with increasing amounts of the ΔTRAP-1 expression vector progressively decreased the induction of p3TP-Lux. Using 600 ng of this TRAP-1 construct, only 12-fold induction was seen (12.2 ± 1.4; p = 0.0001 versus the absence of TRAP-1). Analogous results were seen following stimulation of endogenous type I receptor with 1 ng/ml TGFβ1. Expression increased to 6.17 ± 0.46, relative to vehicle-treated cells (p = 0.0001), and this induction likewise was inhibited by ΔTRAP-1 (2.51 ± 0.24, using 600 ng; p = 0.0001 versus the absence of TRAP-1). Thus, a partial TRAP-1 cDNA, which is sufficient to recognize the activated receptor selectively, can function as an inhibitor of TGFβ-dependent gene transcription.

DISCUSSION

Ligand-induced receptor homodimerization, causing reciprocal cross-phosphorylation in trans, is a strategy commonly employed by receptor tyrosine kinases for transmembrane signaling (27). Phosphorylated tyrosine residues of the receptor, in turn, recruit SH2 domain-containing molecules that are required for signaling. For receptor serine/threonine kinases and the TGFβ superfamily, the signal transduction pathway is similar but distinct; TGFβ ligands induce the heterodimerization of TβR-II and TβR-I, whereas TβR-II phosphorylates TβR-I on serine and threonine residues of its Gly/Ser-rich domain. This directional phosphorylation event and the kinase activity of both receptors functioning sequentially are required for signal generation (1). Conversely, we and others have shown that negatively charged amino acids can serve as surrogates for the phosphorylation of TβR-I and constitutively activate the receptor even in the absence of TβR-II (9, 28).

According to this model, TβR-I, being the substrate for TβR-II, should interact with downstream mediators and effectors. Therefore, it has been discouraging that interaction cloning using the TβR-I cytoplasmic domain as bait has not succeeded to date in identifying specific molecules that confer the TβR-I signal (3, 5, 7, 29). Based on precedents with receptor tyrosine kinases, one foreseeable explanation for this failure may be that some effector molecules acting downstream of TβR-I might associate preferentially with phosphorylated (active) TβR-I rather than with the unphosphorylated (inactive) TβR-I used as bait in previously reported cloning efforts. Here, we have used TβR-I 



P194A.A204D as bait, which has two potentially advantageous properties for this purpose: being autonomously active and not binding FKBP12. Beyond its ability to distinguish activated from wild-type TβR-I in yeast two-hybrid assays, the novel protein, TRAP-1, binds only mutationally activated or ligand-activated TβR-I in mammalian cells not the inactive type I receptor, reminiscent of the signaling tactics used by receptor tyrosine kinases. This novel protein is selective in binding active forms of TβR-I, which favors its involvement in the TGFβ cascade. Similar binding specificity recently was demonstrated for Smad7, which preferentially associates with active TβR-I, preventing receptor association with and phosphorylation of Smad2 (30). Thus, intracellular antagonists as well as mediators might be identified by use of the activated receptor as bait. Indeed, at least for Smad proteins, only the inhibitory forms like Smad7 have been shown to bind stably to the activated receptor. In keeping with this model, the C-terminal portion of TRAP-1 (which is sufficient for stable interaction with the activated receptor both in yeast and in mammalian cells) was found to inhibit TGFβ signaling, as measured by the p3TP-Lux reporter gene. This does not preclude a more complex role for the full-length protein, which we have not yet expressed, or address the status of TGFβ signaling in the absence of TRAP-1. Further work is required to validate the suggested function of TRAP-1 in TβR-I signaling via Smad, TAK1, or an alternative pathway.

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