The Type II Transforming Growth Factor (TGF)-β Receptor-interacting Protein TRIP-1 Acts as a Modulator of the TGF-β Response*

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The transforming growth factor-β (TGF-β) receptor interacting protein TRIP-1 was originally identified as a WD40 repeat-containing protein that has the ability to associate with the TGF-β type II receptor and is phosphorylated by it (1). However, its function was not known. We now show that TRIP-1 expression represses the ability of TGF-β to induce transcription from the plasminogen activator inhibitor-1 promoter, a common reporter of the TGF-β-induced gene expression response, but does not affect the ability of TGF-β to inhibit cyclin A transcription. TRIP-1 can also inhibit the plasminogen activator inhibitor-1 expression induced by Smads as well as activated TGF-β type I receptors. Its inhibitory effect is exerted by a combination of receptor-dependent and receptor-independent mechanisms. Deletion mutational analysis revealed that two distinct regions, which do not contain recognizable WD40 repeats, are required for the ability of TRIP-1 to inhibit the gene expression response. Expression of other segments of TRIP-1 increased the TGF-β-induced gene expression response and therefore may exert a dominant negative phenotype. We conclude that TRIP-1 acts as a modulator of the TGF-β response.

Transforming growth factor-β (TGF-β)1 is the prototype member of a superfamily of growth factors, which have many roles in growth regulation, wound healing, immunity, and development. Most notably, TGF-β is a potent growth inhibitor for many cell types and induces a variety of extracellular matrix proteins and adhesion receptors (2). The current model for TGF-β signaling invokes the binding of ligand to the type II TGF-β receptor, which results in the recruitment, phosphorylation, and concomitant activation of type I TGF-β receptors. The activated type I receptor then phosphorylates Smad2 or Smad3, which is then released from the receptor and forms a complex with Smad4. This complex then translocates to the nucleus where it interacts with transcription factors and regulates gene expression (3).

Although Smads clearly act as effectors of TGF-β receptor signaling, several lines of evidence indicate that other factors are probably involved in TGF-β signal transduction. The ability of a dominant negative type II receptor to inhibit TGF-β-mediated growth inhibition but not extracellular matrix production suggests that the pathways leading to these effects of TGF-β are distinct and separable (4) and may be differentially regulated via quantitatively different thresholds of signaling (5). Consistent with this notion, specific residues on the type I (6) or type II (7) receptor have been identified that are dispensable for TGF-β stimulation of extracellular matrix production but required for the antimitogenic effect. The Smads have been found to mediate both the extracellular matrix and the antimitogenic signaling pathways (3, 8, 9), and no evidence yet presented has indicated a role for Smads in differentially modulating distinct TGF-β signaling pathways. Thus, although the basis for distinction between these pathways is not well understood, it is possible that other proteins interacting directly with the receptors are involved in this differential regulation.

In addition to Smads, other proteins have been identified that interact with the TGF-β receptors. Two-hybrid screening approaches resulted in the cloning of FKBP-12 (10) and the θ subunit of farnesyltransferase (11, 12), which interact with the type I receptor, and TRIP-1, a WD40 repeat-containing protein, which interacts with the type II receptor (1). However, farnesyltransferase activity has since been shown to be dispensable for TGF-β signaling, and TGF-β does not affect the activity of this enzyme (13); therefore the functional significance of the interaction of θ subunit of farnesyltransferase with TGF-β receptors is unknown. FKBP-12 has been found to have an inhibitory effect on signaling through TGF-β family type I receptors (14, 15) and can inhibit phosphorylation of the type I receptor by the type II TGF-β receptor (15). No function for TRIP-1 has as yet been reported.

Unlike θ subunit of farnesyltransferase and FKBP-12, TRIP-1 does not appear to have any enzymatic function, a common phenomenon among WD40 repeat-containing proteins (16). WD40 repeats, first recognized in the β subunit of heterotrimeric G proteins (17), have been identified in many proteins involved in diverse functions, such as transcriptional regulation, RNA processing, signal transduction, cell cycle progression, and vesicular trafficking (16). These proteins are often found in multi-subunit protein complexes, and it has been proposed that the WD40 repeats are important for protein-protein interactions. The crystal structure of Gβγ has revealed the three-dimensional structure of the WD40 repeat units in Gβ as forming a β-propeller structure, with each propeller blade being formed from a four-strand β-sheet (18). Subsequent studies suggest that other WD40 repeat proteins are likely to form similar propeller structures (19).

TRIP-1 interacts with the type II TGF-β receptor both in vivo and in vitro but does not interact with the closely related type II activin receptor. The kinase activity, and therefore, presum-
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ably, autophosphorylation of the receptor greatly enhance this interaction, and TRIP-1 is a substrate of the receptor kinase. TRIP-1 can be found in the ligand-bound complex of type I and II receptors but does not interact directly with the type I receptors (1). Recently, another WD40 repeat-containing protein has been identified that interacts with TGF-β receptors. Ba, a subunit of protein phosphatase 2A, interacts with type I TGF-β receptors and potentiates the anti-mitogenic signaling pathway in a receptor-dependent manner. However, Ba has little or no effect on PAI-1 expression induced by the activated receptor complex, suggesting that Ba does not affect TGF-β action. 

To generate the ΔM construct, in which amino acids 104–325 are deleted, the ΔM construct was digested with MrqI and XhoI, the insert was removed, and the synthetic oligonucleotides 5′-CCGGAGTCGACGTCGACGTCGCTGTTTTCGTAAG and 5′-GGCGGCCAAGCTTGGTTTGATTTTG and 5′-GGCGGCCAAGCTTGGTTTGATTTTGA and 5′-GGCGGCCAAGCTTGGTTTGATTTTG were annealed into a single piece of double-stranded DNA encoding amino acids 98–103, were cloned in place of the insert.

To generate the ΔC construct, in which amino acids 1–90 and 227–325 are deleted, the ΔC construct was digested with MrqI and XhoI, the insert removed, and the synthetic oligonucleotides 5′-ATGCTGCAGCCATGCGGTTTTGA and 5′-TTCTATCGATTGAATTCAGC and 5′-TTCTATCGATTGAATTCAGC and 5′-TTCTATCGATTGAATTCAGC, which were annealed into a single piece of double-stranded DNA encoding amino acids 98–103, were cloned in place of the insert.

To generate the ΔN construct, in which amino acids 1–90 and 227–325 are deleted, the ΔN construct was digested with MrqI and XhoI, the insert removed, and the synthetic oligonucleotides 5′-ATGCTGCAGCCATGCGGTTTTGA and 5′-TTCTATCGATTGAATTCAGC and 5′-TTCTATCGATTGAATTCAGC, which were annealed into a single piece of double-stranded DNA encoding amino acids 98–103, were cloned in place of the insert.

To generate the ΔM construct, in which amino acids 1–90 and 97–226 are deleted, the ΔM construct was digested with MrqI and XhoI, the insert was removed, and the synthetic oligonucleotides 5′-CCGGAGTCGACGTCGACGTCGCTGTTTTCGTAAG and 5′-GGCGGCCAAGCTTGGTTTGATTTTG were annealed into a single piece of double-stranded DNA encoding amino acids 98–103, were cloned in place of the insert.
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RESULTS

The two best characterized responses to TGF-β receptor activation are the induction of extracellular matrix protein genes and growth inhibition. Both responses can be used to evaluate receptor function using transient transfection assays, whereby the levels of luciferase expression, directed from defined promoters, are measured in response to TGF-β. Transcription of a luciferase reporter from the PAI-1 promoter segment is frequently used to measure induction of extracellular matrix protein synthesis (27, 28), whereas the inhibitory effect of TGF-β on cell proliferation can be conveniently assessed by the decrease of luciferase expression from the cyclin A promoter (26). To determine whether TRIP-1 plays a role in TGF-β signaling, we used these transient transfection assays to assess the effect of increased TRIP-1 expression from an expression plasmid on luciferase expression from a cotransfected luciferase reporter plasmid, in the absence or presence of TGF-β. Transcription of the PAI-1-luciferase reporter p800luc, which increases in response to TGF-β treatment (27), was measured in the presence or absence of a cotransfected TRIP-1 expression plasmid. As shown in Fig. 1A, increased TRIP-1 expression inhibited transcription from the PAI-1 promoter and strongly repressed its stimulation by TGF-β in the human HaCaT keratinocyte cell line. Similar data were obtained in Mv1Lu cells (data not shown and Figs. 3, 4, 6, and 7). The moderate inhibition of transcription from the PAI-1 promoter in the absence of exogenous TGF-β was not due to a general inhibition of transcription, because the luciferase expression levels were normalized to an internal control (β-galactosidase expression), and, furthermore, TRIP-1 overexpression did not result in a similar decrease in β-galactosidase expression (data not shown). In contrast to the effect of TRIP-1 on expression from the PAI-1 promoter, the luciferase expression from the cyclin A promoter was not significantly affected by increased TRIP-1 expression (Fig. 1B). Our results therefore indicate that TRIP-1 expression inhibits the TGF-β response, but selectively affects the induction of PAI-1 gene expression and not the expression from the cyclin A promoter. This differential responsiveness may reflect a selectivity for the gene induction response, without affecting the growth inhibitory response.

To determine whether the inhibitory effect of TRIP-1 depends on TGF-β receptor function, we examined its activity on expression from the PAI-1 promoter in the absence of functional heteromeric TGF-β type II/type I receptor complexes. For this purpose we used the cell line DR26, a mutant cell line derived from Mv1Lu cells, which lacks functional type II receptors (29). In DR26 cells, TRIP-1 caused a moderate inhibition of the transcription from the PAI-1 promoter (Fig. 2), but this decrease was less than the TRIP-1-mediated inhibition of PAI-1 expression in wild-type Mv1Lu cells in the absence of TGF-β. Introduction of the type II receptor by transfection resulted not only in the acquisition of TGF-β responsiveness but also in a strong inhibition of PAI-1 expression (Fig. 2). These data indicate that TRIP-1 strongly inhibits the TGF-β-induced PAI-1 expression and suggest that although the inhibitory effect of TRIP-1 in wild-type Mv1Lu cells without TGF-β treatment may be due in part to a decrease in autocrine signaling by endogenous TGF-β, consistent with previous observations (30).

Because TRIP-1 interacts with the type II but not the type I receptor (1), the possibility exists that TRIP-1 expression might not inhibit PAI-1 expression induced by a constitutively activated type I TGF-β receptor. Indeed, a mutant TβRI type I receptor has been described in which Thr at position 204 has been replaced by Asp, i.e. the T204D mutant (24), thus resulting in increased activity. However, whereas this mutant type I receptor has a higher basal activity than the wild-type I receptor, ligand binding to the type II receptor and subsequent transphosphorylation of type I by the type II receptor still enhances its activity considerably (24, 31). Thus, we expressed the mutated TβRI (T202D for the rat version of TβRI) into Mv1Lu cells and assessed the effect of TRIP-1 on the PAI-1 response. As shown in Fig. 3A, the T202D type I receptor induced a ligand-dependent response, and TRIP-1 expression exhibited a strong inhibition of transcription from the PAI-1 promoter in these cells. Because this inhibition may be due to the dependence of the signaling by the mutant TβRI on functional type II receptors, consistent with previous findings (31), we assessed the effect of TRIP-1 on induction of the PAI-1 response in the type II receptor-defective DR26 cells. In these
cells, the activated type I receptor exhibited ligand independence and could therefore be considered to be independent of the type II receptor for its action. Under these conditions, the ability of TRIP-1 to inhibit signaling to PAI-1 was severely curtailed (Fig. 3B), suggesting that the inhibitory activity of TRIP-1 depends to a considerable extent on a functional type II receptor and TGF-β receptor complex.

We next examined the ability of TRIP-1 to inhibit the induction of transcription from the PAI-1 promoter by Smad3 and 4, which act as effectors of TGF-β signaling following receptor activation (3, 8, 9). However, although these signal transducers act downstream from the receptors, and their co-overexpression induces a ligand-independent response, they nevertheless still exhibit receptor dependence. Thus, their activity is inhibited by overexpressing a dominant negative mutant of a TGF-β receptor and enhanced by overexpressing both the type II and type I receptors (9). Our results indicate that increased TRIP-1 expression inhibited transcription from the PAI-1 promoter by Smad3 and 4 (Fig. 4A). The inhibition of the Smad3/4-induced transcription by TRIP-1 was somewhat less effective in DR26 cells than in wild-type Mv1Lu cells (Fig. 4B), suggesting that the presence of functional TGF-β receptor complexes contribute to the ability of TRIP-1 to inhibit the Smad3/4-induced transcription.

To analyze which domains of TRIP-1 are required for its inhibitory effect on TGF-β receptor signaling to the PAI-1 expression response, we expressed several deletion mutants of the protein (Fig. 5). Three different plasmids expressed mutant TRIP-1 proteins, each with a different segment deleted that corresponds to about one-third of the protein. These mutants, ΔN-Myc, ΔC-Myc, or ΔM-Myc, lacked the N-terminal, C-terminal, and middle thirds of the protein, respectively, including one or two WD40 repeats (Fig. 5). Three other mutants, ΔMC-Myc, ΔNM-Myc, or ΔNC-Myc, corresponded to the segments that were deleted in the previous three mutants. Finally, the ΔWD-Myc mutant had all WD40 repeats deleted, thus leaving only the two intervening regions plus short N- and C-terminal extensions (Fig. 5).

The effect of the different TRIP-1 deletion mutants on TGF-β receptor-induced expression from the PAI-1 promoter was then tested in transient transfection assays in Mv1Lu cells. As shown in Fig. 6, the ΔN-Myc and ΔWD-Myc mutants of TRIP-1 exhibited an inhibitory activity on luciferase expression from the PAI-1 promoter, thus resembling the effect of full-length TRIP-1 protein, whereas the ΔC mutant appeared to lack inhibitory activity. This lack of activity of the ΔC mutant compared with the ΔN mutant was not due to a difference in the level of expression of the protein (data not shown). In contrast, expression of other deletion mutants of TRIP-1 enhanced the TGF-β response, and this increase was strongest with the ΔNC-Myc and ΔNM-Myc mutants. Because TRIP-1 is endogenously expressed in most if not all cell types, including Mv1Lu (1) and wild-type TRIP-1 inhibits the TGF-β response, the increased TGF-β response by the latter two mutants suggests that they act as dominant negative inhibitors of endogenous TRIP-1 function. Consistent with the moderate inhibitory effect of full-length TRIP-1 in the absence of exogenous TGF-β,
these dominant negative inhibitors also increased the basal level of transcription from the PAI-1 promoter. Interestingly, the mutants, which inhibited the TGF-β response similarly to wild-type TRIP-1, contained the two protein segments of TRIP-1 that separate the WD40 repeat regions. The dominant negative mutants have only one or none of these intervening segments, and the strongest interference with endogenous TRIP-1 activity is exerted by mutants that lack the N-terminal two WD40 repeats. Surprisingly, because the ΔWD-Myc mutant inhibits the TGF-β-induced PAI-1 response, the WD40 repeat regions of the protein are apparently not required for the inhibitory effect of TRIP-1. The differential effects of the deletion mutants of TRIP-1 on the PAI-1 response did not reflect an inability of some of these proteins to interact with the type II TGF-β receptor. Indeed, when the various Myc-epitope-tagged TRIP-1 mutants were coexpressed with Flag-tagged type II receptor, the type II receptor was always coprecipitated in the TRIP-1-specific anti-Myc immunoprecipitations (Fig. 7), although differences in affinity cannot be excluded. In addition, under these experimental conditions, the protein phosphatase 2A subunit Bα, which also contains five WD40 repeats, does not associate with the type II receptor, whereas it does associate with type I receptors, thus supporting the specificity of the TRIP-1 interaction with the type II receptor. We therefore conclude that all of the mutants tested retained some ability to interact with the type II TGF-β receptor.

Fig. 4. TRIP-1 inhibits the Smad3/4-induced PAI-1 response. Wild-type MvxLu (A) or DR26 cells (B) were transfected with the p800luc reporter plasmid with or without expression plasmids for Smad3 and/or TRIP-1 expression plasmids. Luciferase expression, normalized for expression of β-galactosidase from the cotransfected pSVβgal, was assessed in the presence or absence of TGF-β.

Fig. 5. Schematic representation of various TRIP-1 deletion mutants, drawn to scale. Deleted regions are indicated by dashed lines.
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DISCUSSION

We have previously shown that a novel WD40 repeat protein, TRIP-1, associates with the type II TGF-β receptor and not with type I receptors (1). In the current report, we have addressed its role in TGF-β receptor signaling. Our data indicate that increased expression of TRIP-1 decreases the TGF-β response, as assessed by induction of transcription from the PAI-1 response. Thus, TRIP-1, which associates with the type II TGF-β receptor, functions as a modulator of the TGF-β response, and we have no evidence that it acts as an effector of TGF-β receptor signaling. The modulatory role of TRIP-1 may thus resemble the effect of FKBP-12, which similarly to TRIP-1 decreases TGF-β responsiveness (15). In contrast with TRIP-1, however, FKBP-12 associates with the type I and not with the type II receptors (10, 15).

TRIP-1 overexpression preferentially inhibits TGF-β-induced gene expression from the PAI-1 promoter and has very little, if any, effect on TGF-β-induced growth inhibition, as assessed by transcription from the cyclin A promoter. The preferential inhibition of one response and not another stands in contrast with the ability of FKBP-12 to decrease both responses, induced by activated TGF-β receptors. This ability of TRIP-1 to preferentially modulate one but not another response may sound surprising, because the current model proposes that TGF-β exerts its multiple activities through a single heteromeric complex of type II and type I receptors and does not invoke differential regulation. Yet, several reports show evidence of distinction between the growth inhibitory effect of TGF-β and its ability to induce gene expression. Indeed, overexpression of a cytoplasmically truncated type II TGF-β receptor has been shown to block the growth inhibitory response without affecting the ability of TGF-β to induce the expression of several genes (4). Furthermore, defined mutations in the type II (7) or type I (6) TGF-β receptors affect the growth inhibitory response to TGF-β but not the ability of TGF-β to induce PAI-1 expression. Finally, increased type I receptor expression results in an increased gene induction response, whereas the growth regulatory response is down-regulated (32). In addition to and in contrast with our findings on TRIP-1, we have recently shown that interaction of another WD40 repeat protein, the Bα subunit of protein phosphatase 2A, with the type I receptor enhances the growth inhibitory response to TGF-β yet has little effect on TGF-β-induced gene expression.2 Thus, both WD40 repeat proteins that interact with TGF-β receptors appear to preferentially regulate one TGF-β response but not the other.

The mechanism by which TRIP-1 inhibits the PAI-1 response is as yet unclear. Our results indicate that although the inhibitory effect of TRIP-1 depends in part on the presence of functional receptors and ligand stimulation, TRIP-1 also has an inhibitory activity that is independent of receptor function and thus downstream from the receptors. The moderate receptor-independent inhibition by TRIP-1 is illustrated by the decrease in PAI-1 expression in DR26 cells, which lack functional type II receptors. The decrease in basal PAI-1 expression in wild-type Mv1Lu cells transfected with TRIP-1 is stronger than in DR26 cells and most likely combines the receptor-independent contribution and a contribution associated with the documented autocrine receptor stimulation by endogenous TGF-β (30). Conversely, the increased basal PAI-1 expression in Mv1Lu cells transfected with a dominant negative TRIP-1 mutant most likely also reflects this combined effect.

An epistatic characterization of how TRIP-1 inhibits TGF-β-induced signaling cannot easily done, because of the very nature of TGF-β signaling. In principle, overexpression of a constitutively active type I receptor might determine to what extent TRIP-1 exerts its activity in the absence of the type II receptor. However, the activity of such mutant TβRI still depends on the activity of the type II receptor (24, 31), and the decreased ability of TRIP-1 to inhibit the mutant TβRI in the absence of the type II receptor indicates that the effect of TRIP-1 on the PAI-1 response is in part dependent on a functional TGF-β receptor complex. Like TRIP-1, the activity of the Smads also exhibit characteristics indicative of both receptor dependence and receptor independence (9). Thus, it is difficult to determine whether the effect of TRIP-1 on the Smad3/4-induced PAI-1 response reflects a receptor-dependent or -independent response. Various other approaches have been used to characterize the mechanism of inhibition of receptor signaling by TRIP-1. However, we have been unable to demonstrate any effect of increased TRIP-1 expression on the availability of the cell surface receptors for binding of 125I-TGF-β, the interaction between the type II receptor with the type I receptor, the overall level of phosphorylation of type I and II receptors, the association of Smad3 with the type I receptor, and its overall phosphorylation and subcellular localization.4 However, because TRIP-1 does not affect all TGF-β signaling equally, its mode of action may not be revealed by examining parameters that merely reflect overall functionality of the TGF-β receptor complex.

The increased TGF-β response in the presence of some TRIP-1 mutants suggests that these mutants exert a dominant negative activity over endogenous TRIP-1, which in turn suggests the involvement of a partner protein. Because WD40 repeat proteins frequently participate in multiprotein complexes (16), it is indeed highly likely that TRIP-1 also fulfills a similar role and thus might function as a bridge between the type II receptor and an as yet unidentified interaction partner. Because the dominant negative mutant versions of TRIP-1 still associate with the type II receptor, these deletions may inactivate their ability to interact with the partner protein. It will therefore be important to identify with which other protein(s) TRIP-1 interacts when associated with the type II receptor. TRIP-1 has recently been identified as a component of the human translation initiation factor complex eIF3 (33), and the yeast TRIP-1 homologue TIF34 has also been found as a subunit of yeast eIF3 (34, 35). Accordingly, sequential immunoprecipitation analyses using TRIP-1 antibodies and an antiserum...
against the eIF3 complex revealed an interaction of TRIP-1 with components of the complex. However, none of the deletion mutants of TRIP-1 was able to associate with the complex.  

Because some of these mutants have an inhibitory activity similarly to wild-type TRIP-1 or exert a presumed dominant negative effect, it is unlikely that the effect of TRIP-1 on PAI-1 expression results from the role of TRIP-1 in the eIF3 complex.

Our results, combined with published evidence, strongly suggest that TRIP-1 may have a dual function, i.e. as a modulator of TGF-β receptor signaling and as a component of the eIF3 complex. This dual function may seem surprising but is not without precedent. The α subunit of eIF2B, another translation initiation factor, interacts not only with the eIF2B complex but also with the cytoplasmic domains of two types of α-adrenergic receptors, and its overexpression causes a small but significant increase in receptor signaling (36). It is unknown if other eIF2 components also interact with the receptor-associated eIF2Bα, but several components that are normally part of the eIF3 complex were found to coimmunoprecipitate with the type II TGF-β receptor.  

Several other examples further illustrate dual functions of components of translation factors. For example, eIF3-p48 is not only a component of the eIF3 complex but also binds to the HTLV1-tax protein and plays a role in its cytoplasmic translocation and furthermore is also involved in regulation of cell proliferation (37, 38). In addition, p16/SUI1/eIF1, which associates with the eIF3 complex and is required for its activity, also exists as a free form whose function is unknown but that does not promote initiation of translation (39).

The structure-function analysis of TRIP-1 identified regions of TRIP-1 that are required for the PAI-1 transcription inhibitory activity and generated mutants that increased the TGF-β response, likely through dominant negative interference with endogenous TRIP-1 function. All these mutants had only one or two WD40 repeats left, in contrast with the full-size and the other mutant forms of TRIP-1, which had more WD40 repeats or none at all (the ΔWD mutant). Furthermore, the two regions in between the WD40 repeats of TRIP-1 were found to be required for its inhibitory activity. However, the mutational analysis did not reveal a defined region of TRIP-1 that corresponded to an interaction domain with the type II receptor. Although these results may seem surprising, they probably reflect the biochemistry and structure of WD40 repeat proteins. Unlike many proteins involved in signaling, which consist of defined, discrete functional modules distributed along the polypeptide chain, WD40 proteins have a propeller-like three-dimensional structure with multiple axes of symmetry. Interaction with partner proteins may well require residues that are distributed at different sites along the length of the protein but that may appear in proximity in the folded protein, as has been shown for TUP-1 and Gjα (40, 41). Some redundancy among WD40 repeats in a protein may also occur; for example, in TUP-1, it has been shown that either repeat 1 or repeat 2 is sufficient for interaction with α2 (42). Finally, WD40 repeat-containing proteins do not necessarily require their WD40 repeats for their functions. A mutant version of TUP-1 that lacks all of its WD40 repeats is still able to carry out many functions similarly to wild type (43, 44). Also, the Arabidopsis WD40 repeat protein COP-1 was found to be very tolerant of extensive deletions, and, in fact, a mutant in which all WD40 repeats were deleted exhibited a much milder phenotype than other mutants with much less extensive mutations in the WD40 repeat sequences (20). Thus, the results from our deletion analysis of TRIP-1 resemble the results obtained with other WD40 repeat proteins, because deletion of all WD40 repeats had much less drastic effects than more restricted deletions and still allowed receptor association and inhibition of the TGF-β response.

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