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TRUSS, a Novel Tumor Necrosis Factor Receptor 1 Scaffolding Protein That Mediates Activation of the Transcription Factor NF-κB

Surinder M. Soond,[1] Jennifer L. Terry,[2] Jeff D. Colbert,[2] and David W. H. Riches[1,2,3]*

Program in Cell Biology, Department of Pediatrics, National Jewish Medical and Research Center, Denver, Colorado 80206,[1] and Department of Immunology[2] and Division of Pulmonary and Critical Care Medicine, Department of Medicine and Department of Pharmacology,[3] University of Colorado Health Sciences Center, Denver, Colorado 80262

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We describe the cloning and characterization of tumor necrosis factor receptor (TNF-R)-associated ubiquitous scaffolding and signaling protein (TRUSS), a novel TNF-R1-interacting protein of 90.7 kDa. TRUSS mRNA was ubiquitously expressed in mouse tissues but was enriched in heart, liver, and testis. Coimmunoprecipitation experiments showed that TRUSS was constitutively associated with unligated TNF-R1 and that the complex was relatively insensitive to stimulation with TNF-α. Deletion mutagenesis of TNF-R1 indicated that TRUSS interacts with both the membrane-proximal region and the death domain of TNF-R1. In addition, the N-terminal region of TRUSS (residues 1 to 440) contains sequences that permit association with the cytoplasmic domain of TNF-R1. Transient overexpression of TRUSS activated NF-κB and increased NF-κB activation in response to ligation of TNF-R1. In contrast, a COOH-terminal-deletion mutant of TRUSS ($TRUSS_{1-723}$) was found to inhibit NF-κB activation by TNF-α. Coprecipitation and coimmunoprecipitation assays revealed that TRUSS can interact with TRADD, TRAF2, and components of the IKK complex. These findings suggest that TRUSS may serve as a scaffolding protein that interacts with TNF-R1 signaling proteins and may link TNF-R1 to the activation of IKK.

Tumor necrosis factor alpha (TNF-α) plays an important role in inflammation, host defense, differentiation, and apoptosis and in the clinical syndromes of septic shock, pulmonary fibrosis, rheumatoid arthritis, type II diabetes, and cachexia. Cellular responses to TNF-α are initiated following its interaction with the receptors TNF-R1 (CD120a) and TNF-R2 (CD120b) (6). Both receptors are members of the TNF receptor superfamily, and while they share significant homology in their extracellular domains, the intracellular domains of these receptors are unrelated. In the case of TNF-R1, ligation by TNF-α or lymphotoxin α induces diverse responses that include the induction of apoptosis following the activation of caspase 8 (4, 38), the transient activation of mitogen-activated protein kinases (9, 52), and the activation of the transcription factors NF-κB and AP-1, which regulate genes involved in acute and chronic inflammation and in innate protection against bacterial infections (28). In addition, NF-κB inhibits TNF-R1-mediated apoptosis by increasing the expression of antiapoptotic genes, including cIAP1, cIAP2, and IEX-IL (49, 53).

NF-κB activation is mediated by the transient activation of an ~600- to 900-kDa IκB-kinase (IKK) complex (36) that is comprised of the catalytic subunits IKKα and IKKβ and the regulatory subunit IKKγ/NEMO (15, 36, 54). IKK in turn catalyzes the phosphorylation of two conserved Ser residues located in the N terminus of IκBα (7) that promote its ubiquitination and degradation by the 26S proteasome (3), thereby uncovering the nuclear localization sequence of NF-κB. The activation of IKK by TNF-R1 is initiated by the recruitment of adapter and signaling proteins to the death domain of the receptor (48). Receptor ligation induces the displacement of SODD from, and association of TRADD with, the death domain of TNF-R1 (24, 25). TRADD in turn serves as an adapter for the binding of RIP and TRAF2 (22, 23) to the receptor complex. RIP, a protein whose presence is essential for the activation of NF-κB by TNF-α, has subsequently been shown to recruit IKKγ/NEMO (58), while TRAF2, a nonessential signaling component (57), interacts with IKKα and IKKβ (14). The mechanism of activation of the IKK complex has been proposed to occur as a consequence of either induced proximity activation following IKKβ oligomerization (41) or through the activity of a MAP3K, possibly MEKK3 (55). Subsequent to these initial signaling events, in which IKK is present as a complex with TNF-R1, the IKK dissociates from the receptor via a process that leads to increased activation of the IKK complex within the cytosol (41, 58).

These findings intuitively raise the question of how the IKK signaling complex is first assembled into macromolecular complexes, stabilized, and then dissociated to allow amplification in the cytosol. In other signaling systems, scaffolding proteins facilitate the assembly of multicomponent signaling complexes (16, 37). For example, JIP-1 and β-arrestin-2 have been shown to assemble components of the JNK signaling pathway (34, 51), while MP1 performs a similar function in the activation of ERK (44). In the TNF-R1 signaling cascade, IKAP, a copurifying component of the IKK complex, was initially suggested to behave as a scaffolding protein (11), although recent work has

* Corresponding author. Mailing address: Program in Cell Biology, Department of Pediatrics, National Jewish Medical and Research Center, Neustadt Room D405, 1400 Jackson St., Denver, CO 80206. Phone: (303) 398-1188. Fax: (303) 398-1381. E-mail: richesd@njc.org.

drawn this conclusion into question (30). Recent studies have also suggested that Hsp90 and Cdc37 are involved in the formation of the IKK complex and are recruited to TNF-R1 upon ligand binding (10). However, the existence and possible role of other scaffolding proteins in TNF-R1 signaling remain to be determined. To address this issue, we conducted yeast two-hybrid screens using the membrane-proximal region (amino acids 207 to 300) of TNF-R1 as a bait. We reasoned that this strategy would result in the identification and cloning of cDNAs encoding proteins which interact with the entire cytoplasmic domain as well as the membrane-proximal region of TNF-R1 and which would be distinct from previously studied proteins that interact primarily with death domains. Several novel protein-coding sequences were cloned, one of which we named TRUSS (for "TNF receptor-associated ubiquitous scaffolding and signaling protein"). As we show here, TRUSS interacts with TNF-R1 and plays a key role in the activation of NF-κB. In addition, we show that TRUSS interacts with TRADD, TRAF2, and subunits of the IKK complex.

MATERIALS AND METHODS

Antibodies. Rabbit antihemagglutinin (anti-HA) antibody (SC805), goat anti-mouse TNF-R1 antibody (SC1070), rabbit anti-TNF-R1 antibody (SC7895), goat anti-TRADD (SC7868), and rabbit anti-TRAF2 (SC877) were from Santa Cruz Biotechnology (Santa Cruz, Calif.). Anti-FLAG M2 (F3165) monoclonal antibody and anti-FLAG M2-agarose (A1205) were purchased from Sigma Chemical Company (St. Louis, Mo.), and anti-Myc monoclonal antibody was from Invitrogen (Carlsbad, Calif.). Hamster monoclonal anti-TNF-R1 antagonist antibody and goat anti-mouse TNF-R1 (AF-425-PB) antibody were from R&D Systems (Minneapolis, Minn.). Hamster antibody HT-57 was a gift from John Freed (National Jewish Medical and Research Center, Denver, Colo.). Anti-TRUSS antisera were raised in rabbits against bacterially expressed GST-TRUSS(1-440) and GST-TRUSS(441-797) by Alpha Diagnostics Inc. (San Antonio, Tex.) and were affinity purified and titrated against recombinant TRUSS.

Two-hybrid screening. The yeast two-hybrid system System-2 and a mouse liver cDNA library were purchased from Clontech Inc. (Palo Alto, Calif.). Competent yeast cells (strain CG-1945) were transformed with 6 μg of p207-300/pAS2-1 bait plasmid and 10 μg of library plasmid DNA and were plated onto minimal synthetic dropout (SD) agar lacking tryptophan, leucine, and histidine but containing 5 mM 3-amino-1,2,4-triazole (Sigma). The plates were incubated at 30°C for 7 days, after which 36 transformants were streaked onto fresh SD agar and tested further for their ability to hydrolyze X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). We also verified that the proteins encoded by prey plasmids did not interact with false baits (GAL4 binding domain-p53 and GAL4 binding domain alone). The two-hybrid-system-derived prey cDNA was used as a probe to clone full-length TRUSS from the mouse liver cDNA library. In addition, a mouse expressed sequence tag clone was obtained from the Japanese Tissue Culture Collection, Tokyo, Japan (clone Mncb-0676; GenBank accession number AU035644). Coding sequences from the transformant-derived prey plasmids were HA epitope tagged and cloned into pcDNA3.1+ to allow expression in mammalian cells. Glutathione *S*-transferase (GST) fusion proteins were created by ligation of TRUSS cDNAs into *Sma*I/*Sal*I- or *Nhe*I/*Xho*I-digested pGEX5x-1 (Amersham/Pharmacia Biotech.). The GST-TNF-R1 fusion protein has been described (12). All GST fusion proteins were expressed in *Escherichia coli* DH5α and immobilized on glutathione-Sepharose-4B (Amersham/Pharmacia Biotech.) as described previously (47).

Transfections and reporter gene assays. NIH 3T3, HEK293 and COS-7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal bovine serum, 100 U of penicillin G per ml, 100 μg of streptomycin per ml, and 2 mM glutamine and were transiently transfected using Lipofectamine or Lipofectamine 2000 reagent (Gibco, Gaithersburg, Md.) as described previously (12). Reporter gene assays for NF-κB activation were conducted following cotransfection with 250 ng of an NF-κB/luciferase construct (a gift from Hong-Bing Shu, National Jewish Medical and Research Center) with an appropriate amount of test construct. The amount of DNA used was kept constant between individual transfections by adding empty vector (pcDNA3.1+). Thirty nanograms of a β-galactosidase-expressing plasmid were also incorporated into the transfection mix to allow the standardization of transfection efficiencies. Unless stated otherwise, cells were incubated for 18 h before being stimulated for 6 h, lysed, and subjected to luciferase activity assays using a luciferase assay system (Promega, Madison, Wis.). The data are means and standard deviations from a representative experiment which was performed a minimum of three times.

Electrophoretic mobility shift assays (EMSAs). Nuclear extracts were prepared from harvested cells as described previously (26), and 4 μg of nuclear protein was incubated with γ-^{32}P-, end-labeled double-stranded consensus oligonucleotides for NF-κB (Promega) for 30 min in binding buffer (20 mM Tris-HCl buffer, pH 7.5, containing 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% [vol/vol] NP-40, and 5% [vol/vol] glycerol). Anti-p65 supershift antibody (2 μg; Santa Cruz Biotechnology) was also incubated with the complexes for 10 min prior to electrophoresis through a 6% polyacrylamide–Tris-borate-EDTA gel. The gels were dried and exposed to hyperfilm MP (Amersham International, Little Chalfont, Buckinghamshire, United Kingdom).

Coimmunoprecipitation and coprecipitation assays. Transfected COS-7 and HEK293 cells or U937 ($\sim 5 \times 10^8$) cells were lysed in HEPES lysis buffer (HLB: 50 mM HEPES buffer, pH 7.6, containing 150 mM NaCl, 5 mM EDTA, 1% [vol/vol] NP-40, 1 mM phenylmethylsulfonyl fluoride, 20 μg of leupeptin per ml, 10 μg of aprotinin per ml, 1 mM NaF, and 1 mM Na_3VO_4). Lysates were centrifuged for 10 min at 20,000 × *g* at 4°C and precleared with protein A/G Plus or protein G Plus Sepharose beads (Santa Cruz Biotechnology), and specific (goat or rabbit anti-mouse TNF-R1) or control antibodies were added to the lysates and incubated at 4°C with rotation for 4 h. The immune complexes were captured with 50 μl of protein A/G PLUS or protein G Plus Sepharose beads at 4°C with rotation overnight, washed in HLB containing 1% (vol/vol) NP-40 for six cycles, boiled in Laemmli sample buffer containing dithiothreitol (20 mM), and resolved by electrophoresis through 10% (wt/vol) sodium dodecyl sulfate (SDS)-polyacrylamide gels followed by Western blot analysis. The procedure for the coprecipitation of transiently expressed proteins was identical to the coimmunoprecipitation protocol described above with the exception that approximately 25 to 30 μg of GST fusion protein bound to glutathione-Sepharose beads was added to the cleared lysate. The mixture was incubated overnight at 4°C, after which bound complexes were washed with HLB containing 0.1% (vol/vol) NP-40 buffer for six cycles.

RESULTS

Cloning TRUSS. To clone proteins that interact with the membrane-proximal region of TNF-R1, we fused the coding region for amino acids 207 to 300 of mouse TNF-R1 to the GAL4 DNA-binding domain of plasmid pAS2-1 as the bait in a yeast two-hybrid screen using a mouse liver cDNA library (17). Thirty-six transformants were obtained, of which 12 proved to be genuine positive transformants by virtue of their failure to interact with irrelevant bait fusion proteins. One of these clones (pHA.TRUSS1-723) was found to encode a protein of ~82 kDa, as inferred from Western blot analysis. This clone was used in a further library screen from which we obtained and determined the complete cDNA sequence. The putative protein sequence of TRUSS was found to span 797 amino acids (Fig. 1A). Translation of the DNA sequence from the ATG start codon to the putative TGA stop codon yielded a predicted molecular mass of ~90.7 kDa, a figure confirmed by (i) Western blotting of whole-cell lysates obtained from TRUSS-transfected COS-7 cells with anti-HA antibody, (ii) immunoprecipitation of lysates from TRUSS-transfected cells with a rabbit polyclonal antibody raised against the NH_2-terminal region (residues 1 to 440) of TRUSS (Fig. 1B), and (iii) Western blotting of endogenous TRUSS in primary cultures of mouse macrophages and several mouse cell lines, including macrophage-like RAW264.7 cells (Fig. 1C). TRUSS was also detected in the human promonocytic cell line U937 (Fig. 1C). We determined the distribution of TRUSS mRNA in mouse tissues and cells by Northern blot analysis. As can be seen in Fig. 1D, TRUSS mRNA exists as a single ~3.2-kb transcript that is widely expressed in mouse tissues, with higher levels

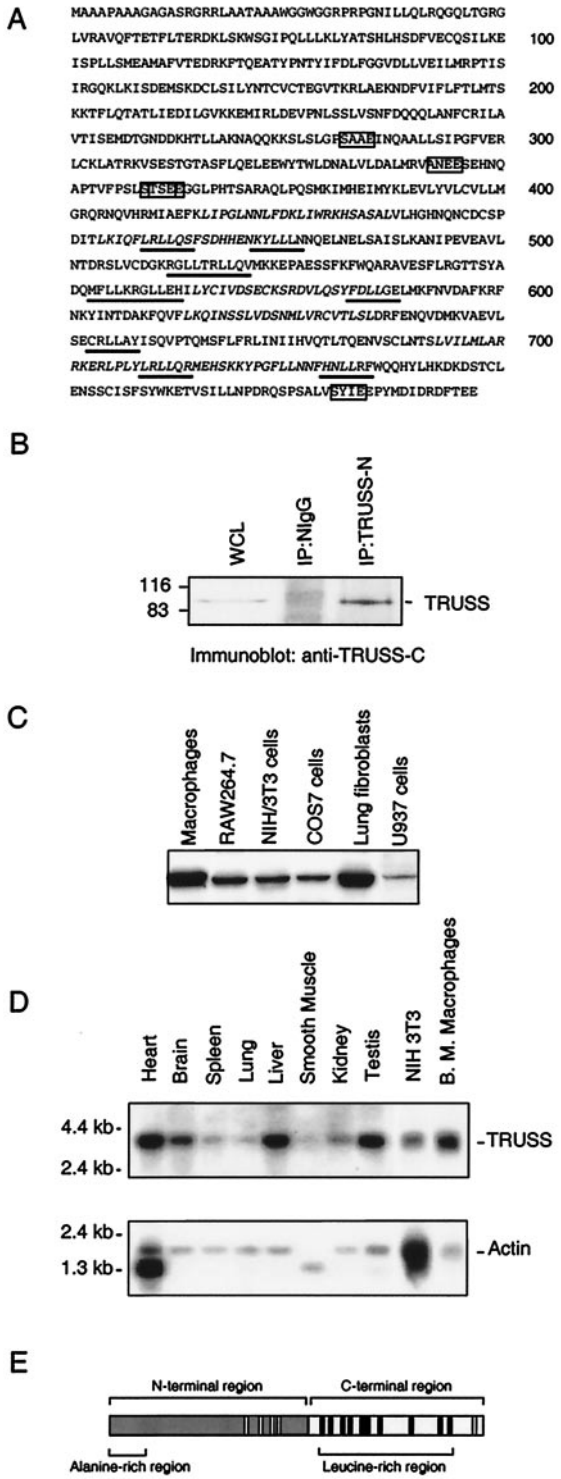

FIG. 1. Primary structure and tissue expression of TRUSS. (A) Predicted amino acid sequence of TRUSS. Consensus TRAF2 binding sequences are boxed, and the predicted dileucine motifs are underlined. Sequences showing similarity to leucine zippers (one mismatch in each sequence) are in italics. (B) Expression of TRUSS protein in transfected COS-7 cells. Approximately 4×10^7 cells were lysed, immunoprecipitated with either rabbit anti-TRUSS NH_2 terminus antibody or nonimmune rabbit IgG (NIgG), and analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting using rabbit anti-TRUSS-COOH antibody. WCL, whole-cell lysate; IP, immunoprecipitation. (C) Expression of endogenous TRUSS in primary cultured mouse macrophages and cell lines. (D) Mouse tissue and cell distribution of TRUSS mRNA as determined by Northern blotting with the full-length TRUSS cDNA. A cDNA probe for β-actin was used to determine RNA loading. B. M. Macrophages, bone marrow macrophages. (E) Schematic showing the proposed organization of TRUSS, the locations of the five predicted TRAF binding regions (white boxes), and the COOH-terminal dileucine repeats (black boxes).

being detected in heart, brain, liver, and testis than in spleen, lung, and kidney. In addition, the 3.2-kb TRUSS transcript was detected in mouse macrophages, NIH 3T3 cells (Fig. 1D), and U937 cells (data not shown).

Database searching revealed no obvious domains within the TRUSS sequence that could implicate a function for this protein in TNF-R1 signaling. Sequence motif searching revealed a number of putative protein Ser/Thr and Tyr kinase phosphorylation sites. Two putative TRAF2 binding sites conforming to the (P/S/A/T)X(Q/E)E consensus (56) and three sites conforming to the SXXE consensus (35, 42) are also present (Fig. 1E). The COOH-terminal region also contains 10 dileucine motifs in which the central dileucine pair is surrounded by one to three charged amino acids (18) and six regions that showed some similarity to the leucine zipper consensus sequence located in the C-terminal region (Fig. 1E). Queries against the mouse genome database indicated that TRUSS is encoded by 20 exons located on chromosome 2.

TRUSS interacts with the cytoplasmic domain of TNF-R1. To determine if TRUSS interacts with TNF-R1 in mammalian cells, we coexpressed HA-tagged TRUSS and FLAG-tagged TNF-R1 in COS-7 cells and conducted coimmunoprecipitation experiments using a nonagonistic hamster monoclonal anti-TNF-R1 antibody followed by Western blotting with anti-HA antibody. As can be seen in Fig. 2A, TRUSS was coimmunoprecipitated with TNF-R1 but not with nonimmune hamster immunoglobulin G (IgG)- or protein A/G-coupled beads. We next determined if endogenous TRUSS was constitutively associated with TNF-R1 or was recruited to the receptor in a ligand-dependent fashion. U937 cells were stimulated with TNF-α for up to 10 min or incubated in medium alone. The cells were then lysed, TNF-R1 was immunoprecipitated, and coimmunoprecipitating TRUSS was detected by Western blotting with an affinity-purified antibody directed against the C-terminal region (residues 441 to 797). TRUSS was found to coimmunoprecipitate with TNF-R1 in the absence of TNF-α, suggesting that TRUSS was preassociated with TNF-R1; however, there was little change in the amount of TRUSS associated with TNF-R1 in response to ligand binding (Fig. 2B, top). As a control, the coimmunoprecipitates were also blotted for TRADD. As expected, TRADD was found to associate with TNF-R1 in the presence but not in the absence of TNF-α (Fig. 2B, bottom). These findings suggest that TRUSS is constitutively associated with TNF-R1 in unstimulated cells and that the level of binding of TRUSS to TNF-R1 is relatively insensitive to ligand binding.

We investigated the ability of both the membrane-proximal region and the death domain of TNF-R1 to bind HA-tagged TRUSS. As shown schematically in Fig. 2C, GST fusion proteins comprising the NH_2-terminal membrane-proximal region

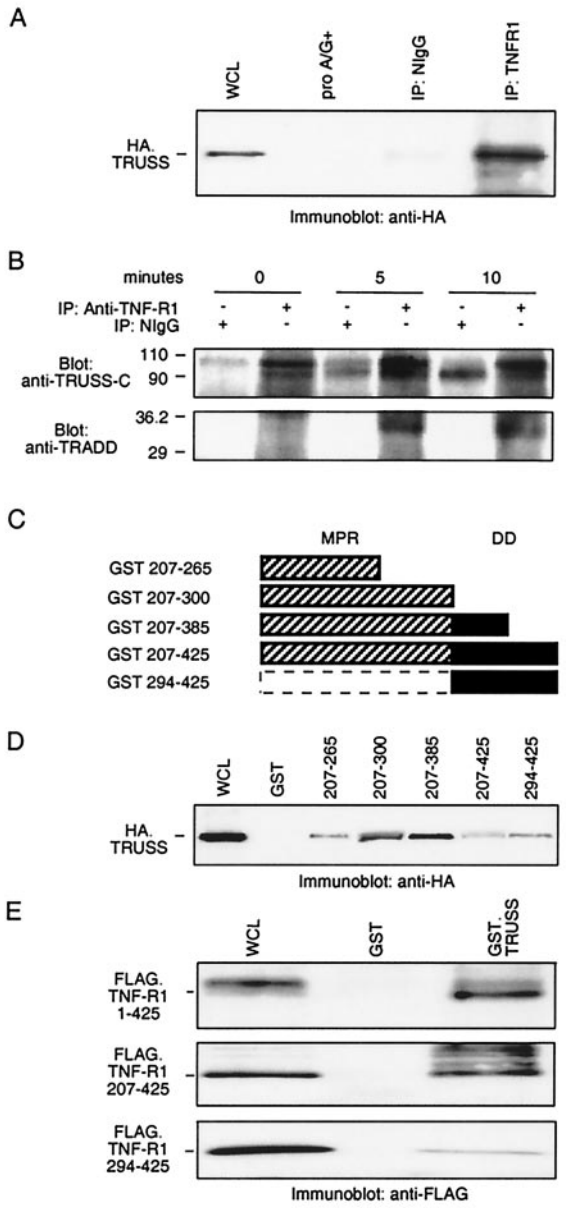

FIG. 2. TRUSS interacts with both the membrane-proximal region and the death domain of TNF-R1. (A) COS-7 cells were cotransfected with pFLAG.TNF-R1 and pHA.TRUSS, lysed, immunoprecipitated with hamster anti-TNF-R1 antagonist antibody, and analyzed by SDS-polyacrylamide gel electrophoresis. TRUSS was detected by Western blotting with anti-HA antibody. WCL, whole-cell lysate; IP, immunoprecipitation; NIgG, nonimmune rabbit IgG. (B) TRUSS interacts with TNF-R1 in U937 cells. Approximately 5×10^8 U937 cells were stimulated with human TNF-α for up to 10 min and lysed. Coimmunoprecipitating TRUSS and TRADD were detected by Western blotting. (C) Schematic representation of the GST-TNF-R1 cytoplasmic domain fusion proteins that were tested for their ability to bind TRUSS. MPR, membrane-proximal region; DD, death domain. (D) Interaction of TRUSS with the cytoplasmic domain of TNF-R1. COS-7 cells were transfected with 3 μg of pHA.TRUSS, lysed, and incubated with equal amounts of glutathione-Sepharose beads coated with the indicated GST–TNF-R1 cytoplasmic domain deletion mutants or GST alone. Coprecipitating proteins were detected by Western blotting with anti-HA antibody. (E) COS-7 cells were transfected with plasmids encoding either (i) FLAG-tagged full-length TNF-R1 (FLAG.TNF-R1 1-425), (ii) the FLAG-tagged TNF-R1 cytoplasmic domain (FLAG.TNF-R1 207-425), or (iii) the FLAG-tagged TNF-R1 death domain (FLAG.TNF-R1 294-425). Lysates were incubated with equal amounts of GST-TRUSS- or GST-coated Sepharose beads, and the coprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting with an anti-FLAG antibody to detect TNF-R1.

(TNF-$R1_{207-265}$ and TNF-$R1_{207-300}$), the COOH-terminal region that included the death domain (TNF-$R1_{294-425}$), and the full-length cytoplasmic domain of TNF-R1 (TNF-$R1_{207-425}$) were coupled to glutathione-Sepharose beads and investigated for their ability to coprecipitate HA-tagged TRUSS from lysates of TRUSS-transfected COS-7 cells. As expected from the results of the two-hybrid screen, HA-tagged TRUSS was found to coprecipitate with fusion proteins containing the membrane-proximal region of TNF-R1 but not with GST alone (Fig. 2D). Also as expected, TRUSS was coprecipitated with the full-length cytoplasmic domain fusion protein (TNF-$R1_{207-425}$). Unexpectedly, TRUSS was also found to coprecipitate with the COOH-terminal region containing the death domain (TNF-$R1_{294-425}$) (Fig. 2D). This finding was further investigated by determining the ability of ectopically expressed FLAG-tagged TNF-R1, FLAG-tagged full-length cytoplasmic domain (pFLAG TNF-$R1_{207-425}$), and the FLAG-tagged COOH-terminal region (pFLAG TNF-$R1_{294-425}$) to bind to GST-TRUSS-coupled glutathione-Sepharose beads. As can be seen in Fig. 2E, full-length FLAG-tagged TNF-R1 as well as both the FLAG-tagged cytoplasmic domain (TNF-$R1_{207-425}$) and to a lesser extent the COOH-terminal region containing the death domain (TNF-$R1_{294-425}$) coprecipitated with GST-TRUSS. In addition, the interaction with the COOH-terminal region of TNF-R1 was confirmed in a two-hybrid screen with a GAL4 DNA-binding domain–TNF-$R1_{294-425}$ construct as the bait (data not shown). These data therefore suggest that TRUSS interacts independently with both the membrane-proximal region and the COOH-terminal region of TNF-R1 that contains the death domain.

TNF-R1 interacts with the N-terminal region of TRUSS. To investigate the region of TRUSS required for the interaction with TNF-R1, we constructed a series of HA-tagged deletion mutants in which the C-terminal region was progressively truncated (as illustrated schematically in Fig. 3A). Plasmids encoding the TRUSS deletion mutants were cotransfected with TNF-R1 into HEK293 cells for 18 h and lysed, and TNF-R1 was immunoprecipitated. Coimmunoprecipitating TRUSS was detected by blotting the immunoprecipitates with anti-HA antibody. As can be seen in Fig. 3B, deletion of the C-terminal region (HA.$TRUSS_{1-440}$) had little effect on the binding of TRUSS to TNF-R1. However, further deletion from the C terminus of HA.$TRUSS_{1-440}$ (as with HA.$TRUSS_{1-192}$) prevented TRUSS from interacting with TNF-R1 (Fig. 3B). In view of these data, we also created a mutant (HA.$TRUSS_{193-440}$) in which both the N-terminal region and the C-terminal region of the basic N-terminal interaction domain were removed. This mutant also failed to interact with TNF-R1 in coimmunoprecipitation experiments (Fig. 3B). These data thus suggest that the N-terminal region encompassing residues 1 to 440 contains a sequence or sequences that facilitate the interaction between TRUSS and TNF-R1.

TRUSS activates NF-κB and increases NF-κB activation by TNF-α. We next investigated the effect of TRUSS on the

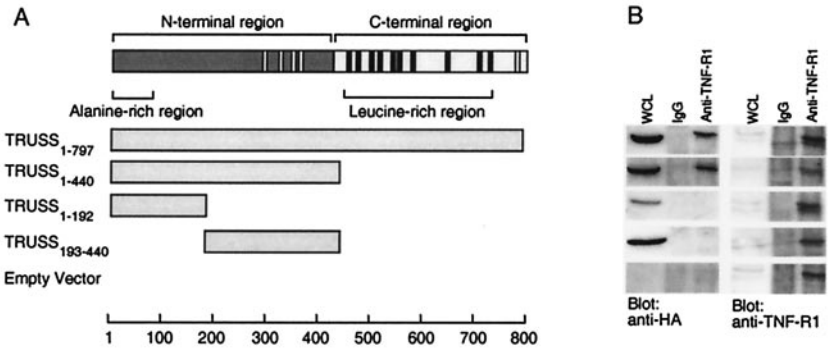

FIG. 3. The N-terminal region of TRUSS interacts with the cytoplasmic domain of TNF-R1. (A) Schematic representation of the HA-tagged TRUSS deletion mutants that were tested for their ability to coimmunoprecipitate with FLAG-tagged TNF-R1. (B) HEK293 cells were cotransfected with 1.0 μg of the indicated HA-tagged TRUSS deletion mutants and 0.5 μg of FLAG-tagged full-length TNF-R1. Lysates were immunoprecipitated with goat anti-mouse TNF-R1 antibody and analyzed by SDS-polyacrylamide gel electrophoresis. Coimmunoprecipitating TRUSS was detected by Western blotting with anti-HA antibody. TNF-R1 was detected in each immunoprecipitate by Western blotting with an anti-TNF-R1 antibody. WCL, whole-cell lysate.

activation of NF-κB using a luciferase reporter gene assay. NIH 3T3 cells were cotransfected with increasing amounts of HA-tagged TRUSS expression plasmid (pHA.TRUSS) and a fixed amount of an NF-κB/luciferase reporter construct for 24 h prior to lysis and analysis of luciferase activity. As can be seen in Fig. 4A (top), transfection with increasing amounts of pHA.TRUSS resulted in a concentration-dependent increase in NF-κB-dependent luciferase expression, with maximum activity (~17-fold activation) being observed upon transfection of 1 μg of pHA.TRUSS. The degree of NF-κB activation was also found to roughly correspond to the level of TRUSS expression as detected by Western blotting of whole-cell lysates from transfected cells (Fig. 4A, bottom). To investigate the effect of TRUSS expression on the activation of NF-κB in response to receptor ligation, NIH 3T3 cells were transfected with pHA.TRUSS for 18 h and were then stimulated with mouse TNF-α (10 ng/ml) for an additional 6 h. Stimulation with TNF-α in the presence of increasing amounts of TRUSS further increased NF-κB-dependent reporter gene expression, with an ~63-fold increase in luciferase activity being observed following stimulation with TNF-α in cells transfected with 1 μg of pHA.TRUSS (Fig. 4A, top). Further increases in the level of TRUSS expression resulted in a diminution in this elevated reporter gene expression.

Previous studies have shown that human TNF-α interacts with mouse TNF-R1 but not TNF-R2, whereas mouse TNF-α binds to both receptors (31). Therefore, to determine if the increase in TNF-α-stimulated NF-κB-dependent luciferase expression observed in the presence of TRUSS was mediated by TNF-R1, NIH 3T3 cells were cotransfected with 0.1 μg of pHA.TRUSS plasmid and the NF-κB reporter plasmid and then stimulated with either human or mouse TNF-α (10 ng/ml). As can be seen in Fig. 4B (top), exposure to human or mouse TNF-α in the absence of transfected TRUSS induced ~7-fold and ~18-fold increases in reporter gene expression, respectively. Stimulation of TRUSS-transfected cells with human TNF-α further increased the level of luciferase expression (~18-fold) compared to that seen in the absence of TRUSS (~7-fold). These findings suggest that the increased level of reporter gene expression seen in the presence of TRUSS is dependent on TNF-R1.

To verify the effect of TRUSS on NF-κB activation, we also performed EMSAs on nuclear extracts prepared from HA.TRUSS-transfected NIH 3T3 cells (Fig. 4C). NF-κB activation was clearly observed in cells transfected with 1 μg of pHA.TRUSS in comparison to cells transfected with empty vector. In addition, the increase in TNF-R1-stimulated NF-κB activation seen in the reporter gene assays in the presence of TRUSS overexpression was also observed in the EMSAs (Fig. 4C). The NF-κB complex was shown to contain p65/RelA, since the complexes formed in both the presence and absence of TRUSS were supershifted in the presence of an anti-p65/RelA antibody (Fig. 4C). Collectively, these findings indicate that TRUSS both stimulates the activation of NF-κB and increases NF-κB activation in response to ligation of TNF-R1.

TRUSS activates NF-κB upstream of RIP and TRAF2. The fact that overexpression of TRUSS both directly activates NF-κB and increases the response to TNF-α could be explained by (i) independent and parallel signaling pathways activated by TNF-R1 and TRUSS, (ii) converging signaling events in which TRUSS utilizes some of the signaling proteins that connect TNF-R1 to IKK, or (iii) an intrinsic role for TRUSS in the TNF-R1 signaling pathway. To begin to distinguish between these possibilities, we coexpressed HA.TRUSS in NIH 3T3 cells together with dominant inhibitory mutants of RIP ($RIP_{559-671}$) (43) or TRAF2 ($TRAF_{287-501}$) (22) in the presence of the NF-κB reporter gene construct. The transfectants were then either incubated in medium alone or stimulated with TNF-α (10 ng/ml), and the lysates were assayed for luciferase activity. As can be seen in Fig. 5A (top), stimulation with TNF-α led to an ~5-fold increase in reporter gene expression in cells cotransfected with empty vector and ~8-fold in cells transfected with pHA.TRUSS. Transfection of $RIP_{559-671}$ led to an expected abolition of TNF-α stimulated NF-κB-dependent reporter gene activation (Fig. 5A, top). However, as can also be seen in Fig. 5A, the increases in reporter gene expression associated with overexpression of TRUSS alone and with the increase in TNF-α-stimulated NF-

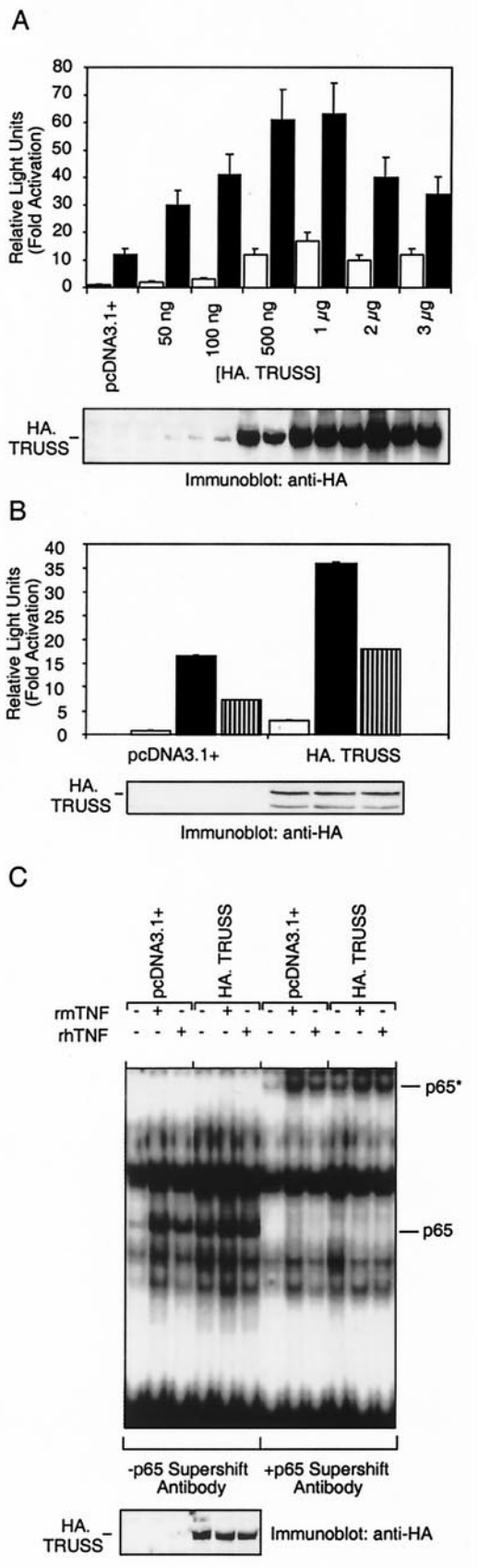

FIG. 4. Transient overexpression of HA.TRUSS activates transcription factor NF-κB in NIH 3T3 cells. (A) NIH 3T3 cells were cotransfected with the indicated amounts of pHA.TRUSS or empty vector and with a fixed amount of the NF-κB luciferase reporter construct. After 18 h, the cells were incubated in the presence (black bars) or absence (white bars) of mouse TNF-α (10 ng/ml) for a further 6 h before lysis and analysis of luciferase activity. (B) NIH 3T3 cells were cotransfected as described above with 0.1 μg of pHA.TRUSS and the NF-κB luciferase reporter construct. The cells were then stimulated with mouse TNF-α (10 ng/ml; black bars) or human TNF-α (10 ng/ml; striped bars) for 6 h or were left unstimulated (white bars) prior to luciferase activity analysis. (C) NIH 3T3 cells were transfected with 1 μg of pHA.TRUSS and then stimulated with mouse TNF-α (rmTNF), human TNF-α (rhTNF) (both at 10 ng/ml), or medium alone for 15 min. NF-κB activity in nuclear extracts was determined by EMSA. p65-containing NF-κB complexes were verified by supershifting with 1 μg of anti-p65 antibody. The lower portion of each panel shows the level of expression of TRUSS as determined by Western blotting. r, recombinant.

κB-dependent luciferase expression following TRUSS overexpression were both reduced to basal levels in cells cotransfected with HA.TRUSS and $RIP_{559-671}$.

Similarly, we cotransfected NIH 3T3 cells with pHA.TRUSS and dominant inhibitory TRAF2 ($TRAF2_{87-501}$) and then investigated the induction of NF-κB-dependent luciferase expression. As seen in Fig. 5B (bottom), stimulation with TNF-α led to an ~9-fold increase in reporter gene expression in cells cotransfected with the empty vector, while an ~15-fold increase in luciferase activity was detected in cells transfected with pHA.TRUSS. The stimulation of NF-κB-dependent reporter gene expression by TRUSS expression alone was largely inhibited in the presence of $TRAF2_{87-501}$, while the increase in signaling seen in TRUSS-transfected cells was almost completely abolished in the presence of $TRAF2_{87-501}$ (Fig. 5B, bottom). Collectively, these findings suggest that both TRAF2 and RIP are required for the activation of NF-κB by TRUSS. In addition, the results place TRUSS proximal to TRAF2 and RIP in the signaling cascade leading to NF-κB activation.

TRUSS is involved in the TNF-α-dependent activation of NF-κB. To investigate the possible role of TRUSS in TNF-α-stimulated NF-κB activation, we created a series of NH_2- and COOH-terminal deletion mutants and assayed them for their ability to disrupt TNF-α-stimulated NF-κB activation following overexpression in NIH 3T3 cells. Figure 6A (bottom) shows that full-length TRUSS and each of the deletion mutants were all expressed in NIH 3T3 cells, as determined by Western blotting of whole-cell lysates with an anti-HA antibody. It can also be seen in Fig. 6A (top) that while each of the deletion mutants inhibited NF-κB-dependent reporter gene expression to some extent, $HA.TRUSS_{1-723}$ exhibited potent dominant inhibitory activity. Transfection with increasing amounts of this construct resulted in a dose-dependent inhibition of reporter gene expression in response to stimulation with TNF-α (Fig. 6B, top), and the degree of inhibition was found to be related to the level of $HA.TRUSS_{1-723}$ expression (Fig. 6B, bottom). These findings support the contention that TRUSS plays an integral role in activating NF-κB in response to stimulation with TNF-α.

TRUSS interacts with TRADD, RIP, TRAF2, and the IKK complex. The finding that NF-κB activation by TRUSS re-

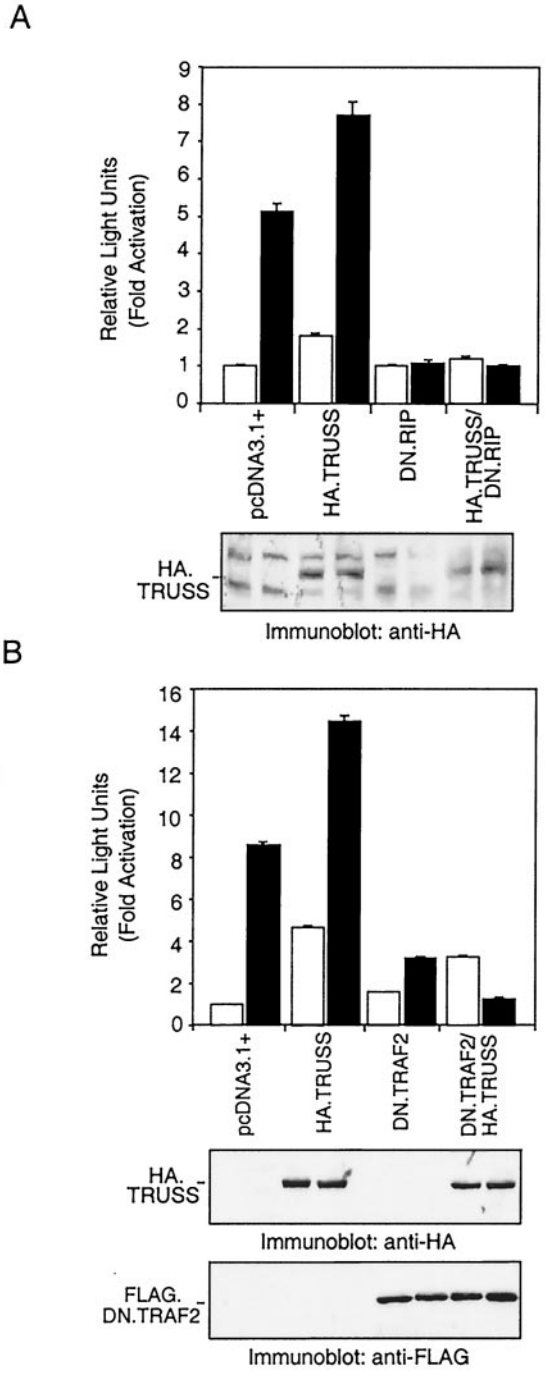

FIG. 5. TRUSS is localized in a proximal position to RIP and TRAF2 in a signaling cascade leading to NF-κB activation. (A) Effect of dominant inhibitory RIP ($RIP_{559-671}$). NIH 3T3 cells were cotransfected as indicated with 1 μg of pHA.TRUSS, $RIP_{559-671}$, or both constructs together with the NF-κB luciferase reporter construct. After 18 h the cells were either stimulated with either mouse TNF-α (10 ng/ml; black bars) or medium alone (white bars) for 6 h prior to luciferase activity analysis. (B) Effect of dominant inhibitory TRAF2 ($TRAF2_{87-501}$). NIH 3T3 cells were cotransfected with 1 μg of pHA.TRUSS, $TRAF2_{87-501}$, or both constructs together with the NF-κB luciferase reporter construct. After 18 h, the cells were stimulated with either mouse TNF-α (10 ng/ml; black bars) or medium alone (white bars) for 6 h prior to luciferase activity analysis. The lower portion of each panel shows the level of TRUSS expression as determined by Western blotting of whole-cell lysates.

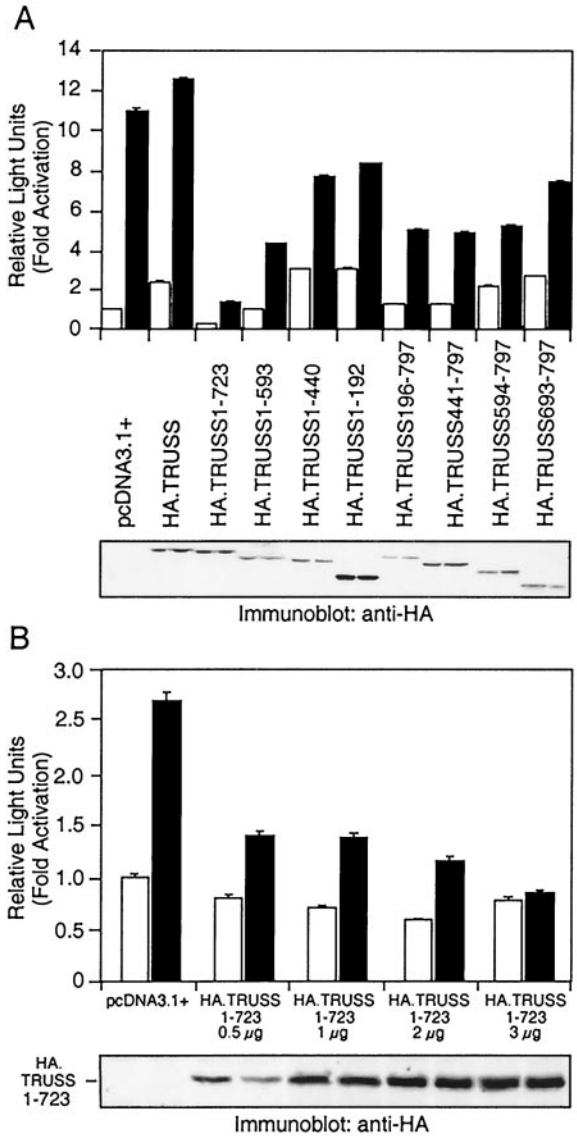

FIG. 6. TRUSS is necessary for TNF-α-stimulated NF-κB activation. (A) NIH 3T3 cells were cotransfected with the 1.0 μg of plasmids encoding the indicated TRUSS deletion mutants and the NF-κB luciferase reporter construct. At 18 h posttransfection, the cells were left unstimulated (white bars) or were stimulated with mouse TNF-α (10 ng/ml; black bars) for 6 h prior to luciferase activity analysis. (B) NIH 3T3 cells transfected with the indicated amounts of pHA.TRUSS1-723 or empty vector. After 18 h the cells were stimulated with mouse TNF-α (10 ng/ml; black bars) or medium alone (white bars) for 6 h prior to luciferase assay. The lower portion of each panel shows the level of expression of TRUSS as determined by Western blotting.

quired TRAF2 and RIP suggested the possibility that TRUSS might be capable of interacting with other signaling molecules (e.g., RIP, TRAF2, TRADD, and the IKK complex) involved in the TNF-R1-dependent activation of NF-κB. Previous studies have shown that TRADD binds to TNF-R1, which in turn binds TRAF2 and RIP via its NH_2-terminal region and death domain, respectively (22–24). Both TRAF2 and RIP have been implicated in the subsequent recruitment of IKKα, IKKβ, and IKKγ to the receptor complex (14, 58). To address this ques-

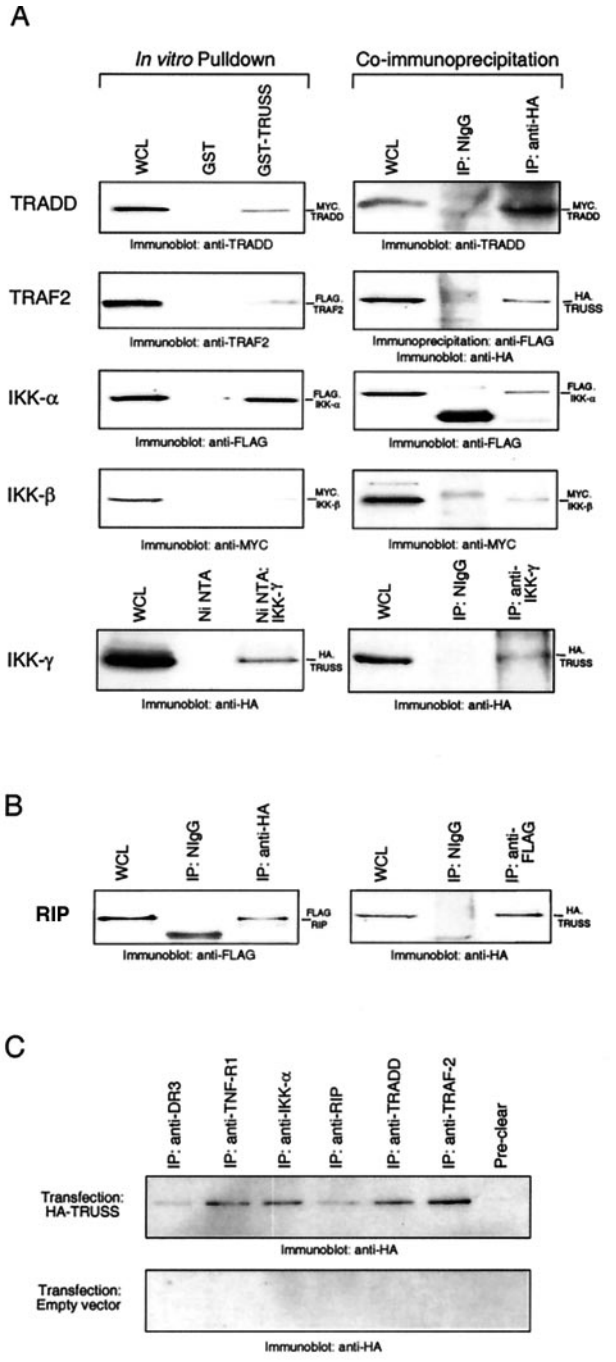

FIG. 7. TRUSS interacts with TRADD, TRAF2, RIP, IKKα, IKKβ, and IKKγ. (A) Interaction of TRUSS with TRADD, TRAF2, and components of the IKK complex. Results of GST- and His-tagged pull-down assays are shown in the left column. COS-7 cells were transfected with plasmids encoding Myc-tagged TRADD, FLAG-tagged TRAF2, FLAG-tagged IKK-α, or Myc-tagged IKK-β, lysed, and incubated with GST-TRUSS or GST-coated Sepharose beads. Interactions between IKK-γ and TRUSS were detected by incubating recombinant His-tagged IKK-γ with lysates from HA-tagged-TRUSS-transfected COS-7 cells. The complexes were captured by binding to Ni-agarose beads. All coprecipitating proteins were detected by SDS-polyacrylamide gel electrophoresis and Western blotting with the antibodies indicated. Results of coimmunoprecipitation assays are shown in the right column. COS-7 cells were cotransfected with plasmids encoding Myc-tagged TRADD, FLAG-tagged TRAF2, IKK-α, IKK-β-, or IKK-γ together with HA-tagged TRUSS, lysed, and immunoprecipitated with anti-HA antibody (or anti-IKK-γ). Coimmunoprecipitating proteins were detected by immunoblotting with the relevant antibody as indicated. WCL, whole-cell lysate; IP, immunoprecipitation; NIgG, nonimmune rabbit IgG; NTA, nitrilotriacetic acid. (B) RIP is present in a complex with TRUSS. COS-7 cells were cotransfected with HA.TRUSS and FLAG-tagged RIP, lysed, and immunoprecipitated with anti-HA antibody or anti-FLAG antibody. Coimmunoprecipitating proteins were detected by Western blotting with anti-HA (TRUSS) or anti-FLAG (RIP) antibodies. (C) TRUSS coimmunoprecipitates with endogenous TRADD, TRAF-2, and IKK in HEK293 cells. HEK293 cells were transfected with HA-tagged TRUSS (top) or empty vector (bottom), lysed, and immunoprecipitated with anti-TNF-R1, anti-TRADD, anti-TRAF2, anti-IKK-α, anti-RIP, or with anti-DR3 antibody as a negative control. Coprecipitating TRUSS was detected by Western blotting.

tion, COS-7 cells were transfected with individual members of the signaling cascade (FLAG-tagged-TRAF2, FLAG-tagged-RIP, FLAG-tagged-IKKα, Myc-tagged-IKKβ, HA-tagged-IKKγ, and Myc-tagged-TRADD), and protein-protein interactions were analyzed by pull-down assays using glutathione-Sepharose beads coated with full-length GST-TRUSS. As can be seen in Fig. 7A (left), TRADD and IKKα were efficiently coprecipitated with GST-TRUSS-coated beads but not with GST-coated beads. TRAF2 and IKKβ were also detected in these coprecipitates, albeit at lower levels than the whole-cell lysates. We were unable to investigate IKKγ binding to TRUSS using the GST-pull down approach because the anti-IKKγ antibody cross-reacted with GST. We therefore incubated lysates from HA-TRUSS-transfected cells with recombinant His-tagged IKKγ and purified the resulting complexes by Ni-agarose affinity chromatography. As can be seen in Fig. 7A (left), TRUSS was efficiently coprecipitated with IKKγ. In contrast, RIP was not bound by GST-TRUSS beads (data not shown).

To confirm these findings we also conducted coimmunoprecipitation experiments in which plasmids encoding HA-tagged TRUSS and the individual signaling molecules (Myc-tagged TRADD, FLAG-tagged RIP, FLAG-tagged TRAF2, FLAG-tagged IKK-α, and Myc-tagged IKK-β and IKK-γ) were cotransfected into COS-7 cells and lysed. TRUSS was then immunoprecipitated with anti-HA antibody, and coimmunoprecipitating proteins were detected by Western blotting with antibodies directed against the appropriate epitope tag. As shown in Fig. 7A (left), TRADD, TRAF2, IKK-α, IKK-β, and IKK-γ were all detected in the coimmunoprecipitates with TRUSS. In addition, RIP was found to be present in a complex with TRUSS as shown by both immunoprecipitation with anti-HA antibody (TRUSS) and blotting with anti-FLAG antibody (RIP) (Fig. 7B). Since RIP was not detected in the pull-down assays using GST-TRUSS, we conclude that the interaction between TRUSS and RIP is indirect. We also determined the ability of ectopically expressed HA-tagged TRUSS to interact with endogenous TRADD, RIP, TRAF2, and the IKK complex in HEK293 cells by immunoprecipitating the endogenous proteins and detecting coimmunoprecipitating TRUSS by blotting with anti-HA antibody. As can be seen in Fig. 7C, TRUSS was found to coimmunoprecipitate with TRADD, TRAF-2, and IKK-α but was only poorly coimmunoprecipitated with endogenous RIP. This pattern of interac-

tion is thus similar to that seen when the TNF-R1 signaling components were overexpressed. Western blotting with antibodies directed against the endogenous TNF-R1 signaling molecules confirmed that they were immunoprecipitated as expected (data not shown). Collectively, these findings suggest that TRUSS is capable of interacting either directly or indirectly with key components of the NF-κB signaling complex, including IKK itself.

DISCUSSION

The molecular mechanisms that connect TNF-R1 to the activation of the IKK complex are now relatively well understood. Current dogma suggests that following its interaction with ligand, the trimerized receptor interacts with TRADD, which subsequently recruits TRAF2 and RIP (22–24). In turn, RIP has been shown to mediate the recruitment of the IKK complex via its interaction with IKKγ (58), while TRAF2 appears to assist in IKK recruitment through its ability to interact with IKKα and IKKβ (14). What remains unclear is how these early events are regulated. In an effort to identify novel proteins that contribute to early events in TNF-R1 signaling, we conducted yeast two-hybrid screens using the membrane-proximal region of TNF-R1. This approach was chosen to avoid the potential bias towards cloning previously identified death domain-interacting proteins that would result from using the entire cytoplasmic domain of TNF-R1. Using this approach, we cloned TRUSS, a ubiquitously expressed protein of 797 amino acids with a molecular mass of 90.7 kDa that was found to interact independently with both the membrane-proximal region and the death domain of TNF-R1. TRUSS lacks a death domain and is also unrelated to other proteins that interact with the TNF-R1 death domain, such as SODD, DAP-1, and sentrin (25, 32, 39). In addition, TRUSS is unrelated to proteins that have previously been shown to interact with the membrane-proximal region of TNF-R1, such as FAN (1), Grb2 (21), TRAP1 (46), 55.11 (5), and PIP5KIIβ (8). Thus, TRUSS is a novel TNF-R1-interacting protein that is unique among such molecules in that it interacts with both the membrane-proximal region and the death domain of TNF-R1.

Several lines of evidence support the notion that TRUSS is involved in signaling events that connect the ligation of TNF-R1 to the activation of the IKK complex. First, TRUSS was coimmunoprecipitated with the unligated receptor, and this association was relatively insensitive to ligand binding. These data suggest that TRUSS may preassociate with the receptor prior to ligand binding or the recruitment of TRADD. Second, transient overexpression of TRUSS was found to increase the activation of NF-κB following stimulation with either human or mouse TNF-α, suggesting shared components in the signaling pathways leading to NF-κB activation. Third, transient overexpression of TRUSS stimulated NF-κB activation, and this response was abolished by cotransfection with dominant inhibitory mutants of both TRAF2 and RIP. These findings suggest that TRAF2 and RIP participate in TRUSS-mediated NF-κB activation. In addition, they place TRUSS in a proximal position in the signaling cascade, a conclusion that is also consistent with the finding that TRUSS interacts with TNF-R1 in two-hybrid and coimmunoprecipitation experiments.

To address the possible role of TRUSS in TNF-α-stimulated, TNF-R1-dependent NF-κB activation, we used deletion mutagenesis to construct and characterize dominant inhibitory TRUSS mutants that were then tested for their ability to disrupt TNF-α-stimulated NF-κB activation. While several constructs expressed some inhibitory activity, $TRUSS_{1\text{-}723}$ was found to be a potent inhibitor of TNF-R1-dependent NF-κB activation. This finding, together with the increase in NF-κB activation observed in response to stimulation of TRUSS-transfected cells with human and mouse TNF-α discussed earlier, supports the conclusion that TRUSS plays a role in connecting TNF-R1 to the activation of the IKK complex. The potent inhibition of NF-κB activation by $TRUSS_{1\text{-}723}$ suggests that the 74 amino acids in the extreme COOH terminus of TRUSS play an important role in the signaling activity of this protein. In contrast, coimmunoprecipitation assays suggested that the N-terminal region of TRUSS (residues 1 to 440) contains sequences that are capable of interacting with TNF-R1.

In view of these findings, we propose that TRUSS may function as a scaffolding protein, and hence we suspected that it would interact with other signaling components that connect TNF-R1 cross-linking to IKK activation. The results of coimmunoprecipitation and coprecipitation studies indicated that TRUSS can interact with TRADD, TRAF2, IKKα, and IKKγ/NEMO and to a lesser extent with IKKβ and RIP. Analysis of the primary structure of TRUSS further supports the contention that TRUSS is a TRAF2-interacting protein, since five potential TRAF2-binding motifs conforming to both major (56) and minor (35, 42) consensus sequences are present, including one (SYIE) located between residues 781 and 784 in the region of TRUSS that is essential for NF-κB activation. However, it is also possible that some of the TRUSS-interacting proteins identified in this study may interact indirectly with TRUSS. For example, we were unable to detect an interaction between RIP and TRUSS using a coprecipitation assay in which GST-TRUSS was bound to glutathione-Sepharose beads, although RIP and TRUSS were detected in a complex when tested in a coimmunoprecipitation assay. The physiologic significance of TRUSS in IKK activation, however, remains to be determined. Perhaps TRUSS assists in IKK recruitment to the TNF-R1 signaling complex. Alternatively, TRUSS may facilitate IKK amplification in the cytosol, perhaps by assisting in the dissociation of IKK from the receptor complex. Ultimately, the creation of a TRUSS-deficient mouse should provide a fuller appreciation of the physiologic role of TRUSS in TNF-R1 signaling.

Primary sequence analysis of TRUSS revealed the presence of multiple dileucine repeats in the COOH-terminal portion of the molecule. Dileucine repeats containing flanking charged residues and/or Ser/Thr phosphorylation sites have been implicated in the internalization and intracellular sorting of receptors and cell surface proteins to the *trans*-Golgi network and to endosomes and lysosomes (18, 19, 29, 40). Subcellular targeting is regulated in part through the interaction of dileucine repeats with the clathrin adapter proteins AP-1, AP-2, and AP-3 (13). While receptors often contain dileucine repeats necessary for internalization, the motif is also present in the COOH-terminal half of the cytoplasmic human immunodeficiency virus type 1 Nef protein. Of significance, this motif has been shown to be critical in the ability of Nef to

stimulate the endocytosis of CD4 through an interaction between the dileucine sequence of Nef and the cytoplasmic domain of CD4 (2, 33). Interestingly, several studies have suggested that the ligand-induced internalization of TNF-R1 is required for the initiation of some signaling events (20, 45, 50). It is currently unclear how the process of internalization may be linked to the current framework of known signaling events though it is conceivable that TRUSS could also participate in TNF-R1 internalization or trafficking to the trans-Golgi network or to the tubular structures to which TNF-R1 has been shown to be localized (12, 27).

In conclusion, we describe the cloning and characterization of a novel signaling protein we have named TRUSS. We show that TRUSS interacts with the cytoplasmic domain of TNF-R1 through binding sites located in both the membrane-proximal region and the death domain of the receptor and in the N-terminal region of TRUSS. In addition, through the creation of a dominant negative mutant of TRUSS we have provided evidence to suggest that TRUSS may participate in the activation of NF-κB by TNF-R1. We have also shown that TRUSS associates with TRADD, TRAF2, and components of the IKK complex, suggesting that TRUSS serves as a scaffolding protein to link TNF-R1 to components of the IKK signalosome.

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