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CD30/TNF receptor-associated factor interaction: NF-κB activation and binding specificity

(TRAF-C domain binding motif)

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ABSTRACT CD30 is a member of the tumor necrosis factor (TNF) receptor superfamily. CD30 is expressed on normal activated lymphocytes, on several virally transformed T- or B-cell lines and on neoplastic cells of Hodgkin's lymphoma. The interaction of CD30 with its ligand induces pleiotropic effects on cells resulting in proliferation, differentiation, or death. The CD30 cytoplasmic tail interacts with TNF receptor-associated factors (TRAFs), which have been shown to transduce signals mediated by TNF-R2 and CD40. We demonstrate here that TRAF2 also plays an important role in CD30-induced NF-κB activation. We also show that TRAF2-mediated activation of NF-κB plays a role in the activation of HIV transcription induced by CD30 cross-linking. Detailed site-directed mutagenesis of the CD30 cytoplasmic tail reveals that there are two independent binding sites for TRAF, each interacting with a different domain of TRAF. Furthermore, we localized the TRAF-C binding site in CD30 to a 5–7 amino acid stretch.

CD30 is a member of the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor superfamily. The TNF/NGF receptor superfamily, which also includes TNF-R1, TNF-R2, the low affinity NGF receptor, CD40, CD27, Fas, 4–1BB, and OX40, is characterized by similar repeating cysteine-rich motifs in their extracellular domains (1–5). Binding of cognate ligands to the receptors causes multiple effects on cells (1–3).

When CD30 is cross-linked it results in cell proliferation, activation of NF-κB, cytokine production, or cell death depending on the cell type (5–9). Although the actual signal transduction pathway mediated by CD30 is not well understood, it has been shown that the C-terminal region of the CD30 cytoplasmic tail is required for both CD30-mediated signaling and interaction with either TRAF1 or TRAF2 (9).

TNF receptor-associated factor (TRAF) proteins were identified in association with their role in the signaling processes mediated by some members of the TNF receptor superfamily (i.e., TNF-R2, TNF-R1, and CD40) or by Epstein–Barr virus LMP1 (10–15). All TRAFs (TRAF1-3) share a highly conserved TRAF domain that mediates receptor interaction and oligomerization (10, 11, 13). The remaining, less conserved, N-terminal region of TRAFs is thought to be involved in the specific signaling processes (10, 11). For example, the deletion of the N-terminal RING finger domain of TRAF2 abolishes its ability to activate NF-κB (13). The highly conserved TRAF domain can be further divided into two subdomains; TRAF-N and TRAF-C (10, 11), each having different binding specificities. TRAF-N is a putative coiled–coil structure that interacts with cellular members of the baculoviral inhibitor of apoptosis protein (c-IAPs) (16), while TRAF-C is a novel protein module that interacts with TNF-R2 or CD40 but not with c-IAPs (11, 16).

In this report we show that CD30/TRAF interaction mediates NF-κB activation, which in turn plays a role in the CD30-mediated activation of HIV transcription. We also show that two regions of the CD30 cytoplasmic tail interact with TRAF1 or TRAF2. The experiments suggested that the N-terminal TRAF binding site of CD30 interacts with a complex site containing elements of both TRAF-N and TRAF-C, whereas the C-terminal TRAF binding site of CD30 interacts with TRAF-C. Furthermore, we defined a 5–7 amino acid stretch of CD30 that was critical for binding to TRAF-C.

MATERIALS AND METHODS

Plasmid Construction. Chimeric receptors expressing the cytoplasmic tail of CD8 (CD8/CD8) or CD30 (CD8/CD30) were previously described (9). The chimeric receptor (CD8/TNF-R2) was generated by in-frame cloning of the cytoplasmic tail of human (h) TNF-R2 into the pCD8Ext, which contains the extracellular and transmembrane portion of CD8α as described (9).

Luciferase reporter constructs containing two Igκ-κB sites (pBIIX-Luc) were previously described (17). Luciferase reporter constructs with the wild-type HIV LTR (pHIV-Luc) or a mutant HIV LTR (pΔκBHIV-Luc) in which both κB sites had been deleted were kindly provided by Kalle Saksela (The Rockefeller University).

The wild-type TRAF1 or TRAF2 expression vectors were made by cloning full-length cDNAs of murine (m) TRAF1 or mTRAF2 (9, 10) into pFJ-AE derived from pSRα-0 (provided by Jae Jung, Harvard Medical School). The N-terminal deletion mutants of TRAF, TRAF1(183–409), and TRAF2(241–501) were made by PCR with 5′ primers containing the translation initiation consensus sequences as described (9), and cloned into pFJ-AE.

Gel-Shift Assay and Antibody Inhibition. Cells were harvested 1 day posttransfection, and nuclear extracts were prepared as described (17). The gel-shift assay was performed as described (17). For antibody inhibition experiments, 5 μg of purified antibodies were incubated with a mixture of nuclear extract and poly(dI-dC) in 1× DNA binding buffer on ice for 30 min before the addition of ^{32}P-labeled κB probe as described (17). The reaction was continued for 15 min at room temperature, and the mixture was loaded onto a 5% native polyacrylamide gel (17).

Transfection Assay. Various expression vectors were transiently transfected into 293 cells by the calcium phosphate

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Abbreviations: TNF, tumor necrosis factor; TRAF, TNF receptor-associated factor; β-gal, β-galactosidase; GST, glutathione *S*-transferase; TBS, TRAF binding site.
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transfection method as described (17). The β-galactosidase (β-gal) reporter construct was cotransfected in all experiments. Two days posttransfection, cell lysates were prepared and assayed for luciferase activity according to the manufacture's protocol (Promega luciferase assay system). The relative luciferase activities were normalized to the β-gal activity.

Interaction of CD30 Cytoplasmic Tail with TRAF1 and TRAF2. The entire cytoplasmic tail or different deletions of CD30 was obtained by PCR as described (9). Site-directed mutagenesis of the CD30 cytoplasmic tail was obtained by PCR with overlapping primers as described (18). Amplified PCR fragments were sequenced and fused in-frame to glutathione *S*-transferase (GST) by cloning into the *Eco*RI and *Not*I sites of pGEX-5X-1 (Pharmacia). Mutation at Ser-342 (Ser → Phe) of the TNF-R2 cytoplasmic tail described in the *Discussion* was fortuitously obtained during PCR and identified by sequencing. The plasmids were transformed into *Escherichia coli*, XL-1 blue (Stratagene). Protein induction, purification and *in vitro* coprecipitation experiments were done essentially as described (9, 14). For *in vitro* coprecipitation experiments, the full-length murine TRAF1 and TRAF2 cDNAs in pBluescript (Strategene) (9, 10) were transcribed and translated *in vitro* using the TNT coupled reticulocytes system (Promega) with ^{35}S-labeled methionine. Equal amounts of *in vitro* translated TRAF1 or TRAF2 were incubated in binding buffer (PBS containing 0.1% Nonidet P-40/0.5 mM dithiothreitol/10% glycerol/1 mM phenylmethylsulfonyl fluoride/2 μg of aprotinin per ml) with ≈1 μg of fusion protein bound to glutathione beads for 45 min at 4°C. After washing five times with binding buffer, the proteins were eluted by boiling in SDS sample buffer for 5 min and subsequently analyzed by SDS/PAGE.

The yeast two-hybrid assays were done essentially as described (19). Different regions of the CD30 cytoplasmic tail were in-frame ligated to LexA-DNA binding domain of pEG202, which were used as bait. Series of N- or C-terminal deletion mutants of TRAF were generated by PCR, sequenced, and in-frame ligated to the transcription activation domain of pJG4-5, which are used as activators. The plasmids and yeast strains for the yeast two-hybrid system were provided by Roger Brent (Harvard Medical School). The interaction of TRAF and CD30 was scored by the β-gal activity of yeast transformants containing both activators and baits upon galactose induction as described (20). In brief, yeast cells were permeabilized with 0.0025% SDS and 5% chloroform, and the cell debris was removed by centrifugation. The β-gal assay was performed at 25°C and OD_{420} was measured (20).

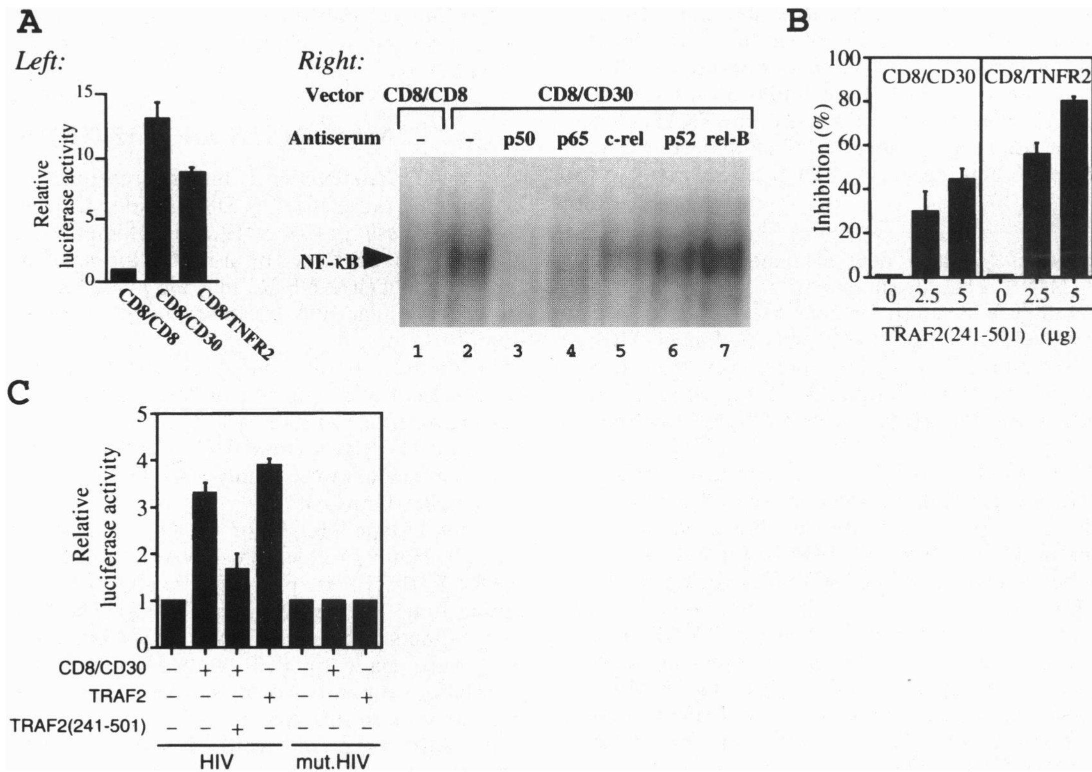


FIG. 1. (*A*) CD30-mediated activation of NF-κB. (*Left*) Chimeric receptors expressing the cytoplasmic domain of CD8 (CD8/CD8), CD30 (CD8/CD30), or TNF-R2 (CD8/TNF-R2) were cotransfected with a luciferase reporter gene plasmid (pBIIX-Luc) containing two Igκ-κB sites (17). Twenty-four hours posttransfection, relative luciferase activities were measured and normalized by β-gal expression as described (17). Average values of triplicate transfection assays from one representative experiment are shown. (*Right*) 293 cells were transfected with chimeric receptors, CD8/CD8, or CD8/CD30, and nuclear extracts prepared from transfected cells were analyzed for the activated NF-κB by gel shift assay as described (17). Gel-shift assays were performed in the presence of preimmune sera (lanes 1 and 2), anti-p50 (lane 3), anti-p65 (lane 4), anti-c-rel (lane 5), anti-p52 (lane 6), and anti-relB (lane 7) as described (17). Major components of the activated NF-κB complexes included p50, p65, and c-rel. (*B*) TRAF2 mediates the CD30-induced NF-κB activation. 293 cells were cotransfected with chimeric receptors (CD8/CD8, CD8/CD30, or CD8/TNF-R2; 1 μg of each plasmid) and pBIIX-Luc. Transfection was also carried out in the presence of increasing amount (2.5 and 5 μg) of TRAF2(241–501)-expression vectors. Percent inhibition was calculated as % inhibition = {[1 − (relative luciferase activity from cells transfected with chimeric receptor and mutant TRAF(241–501)]/(relative luciferase activity from cells transfected with chimeric receptor alone)} × 100. (*C*) TRAF2 mediates the CD30-induced NFκB-dependent activation of HIV expression. 293 cells were cotransfected with chimeric receptors (1 μg) and luciferase gene reporter plasmids (1 μg) containing either the wild-type HIV LTR (HIV) or a mutant HIV LTR lacking κB sites (mut.HIV) and relative luciferase activities were measured as described in *B*. In some cases, expression vector (5 μg) with mutant TRAF2, TRAF2(241–501), was cotransfected with the chimeric receptor, CD8/CD30 (1 μg). TRAF2 expression vectors were also cotransfected with different HIV-luciferase reporter genes described above, and relative luciferase activities were measured.

RESULTS

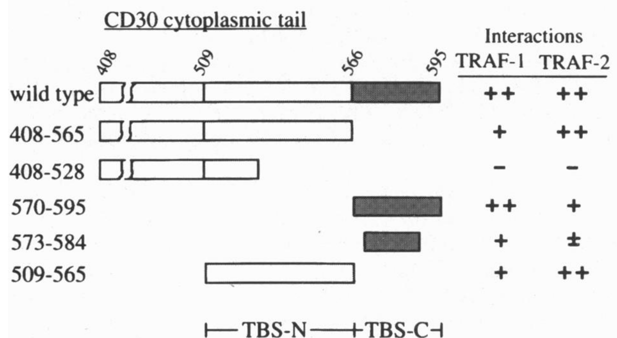
TRAF2 Mediates the Activation of NF-κB and HIV Transcription by CD30. In a recent study (21), it was shown that CD30 cross-linking induces NF-κB activation, which resulted in the transcriptional activation of HIV in some latently infected cell lines. Since the cytoplasmic tail of CD30 interacts with TRAF1 and/or TRAF2 independently (9), we decided to determine the role of TRAFs in the CD30-mediated activation of NF-κB and HIV by using a transient transfection system which was employed to study the signaling pathway mediated by TNF-R2 (13).

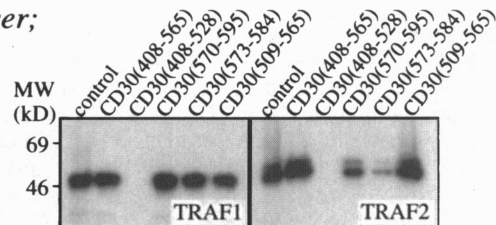
When transiently transfected into 293 cells, the chimeric receptor CD8/CD30 that links the extracellular and transmembrane domains of CD8 to the cytoplasmic tail of CD30 (9) activated NF-κB without the addition of a cross-linking reagent (Fig. 1*A*). Transient transfection of the chimeric receptor CD8/TNF-R2 also activated NF-κB (Fig. 1*A*), which is consistent with the results of transient transfection experiments with the wild-type TNF-R2 (13). Transient transfection of the CD8 receptor did not activate NF-κB in 293 cells (Fig. 1*A*).

To determine which of two TRAFs (TRAF1 or TRAF2) plays a role in CD30-mediated activation of NF-κB, CD8/CD30-expression vector was cotransfected with expression vectors encoding mutant TRAF2 that lacks the N-terminal RING finger domain [TRAF2(241–501)] (Fig. 1*B*). Coexpression of TRAF2(241–501) significantly inhibited NF-κB activation by CD8/CD30 (Fig. 1*B*). However, coexpression of TRAF1 or mutant TRAF1 (expressing residues 183–409) did not affect CD30-mediated activation of NF-κB (data not shown). Coexpression of TRAF2(241–501) also significantly inhibited NF-κB activation by CD8/TNFR2 (Fig. 1*B*), similar to the NF-κB activation by CD8/C30 and also as previously shown (13). These results suggest that TRAF2 is involved in the NF-κB activation by CD30 as well as TNF-R2.

We next examined whether TRAF2 also mediates the activation of HIV transcription by cotransfection experiments of luciferase reporter vectors containing HIV LTR with CD8/CD30 and/or different TRAF expression vectors. CD8/CD30 expression activated the HIV LTR promoter, in which the NF-κB binding sites were required for the observed activation (Fig. 1*C*). The CD30-mediated activation of HIV LTR was significantly inhibited by coexpression of TRAF2(241–501) (Fig. 1*C*). In addition, TRAF2 overexpression alone was sufficient to activate HIV LTR by the NFκB-dependent pathway (Fig. 1*C*). These results suggest that CD30-induced activation of HIV expression is in part mediated by TRAF2.

Identification of Two TRAF Binding Sites in the CD30 Cytoplasmic Tail. Previously we showed that the C-terminal 66 amino acids of CD30 are required for its interaction with TRAF1 or TRAF2 (9). To determine the structural basis for the CD30/TRAF interactions, additional N-terminal and C-terminal deletion mutants of CD30 expressed as GST-fusion proteins were tested for their interactions with TRAF by *in vitro* coprecipitation assays as described (9, 14). A GST-fusion protein expressing only the C-terminal 26-amino acid residues of CD30 [CD30(570–595)] still interacted with TRAF1 and TRAF2 (Fig. 2*A*). However, a GST-fusion protein with mutant CD30 cytoplasmic tail lacking the same C-terminal 26 amino acids [CD30(408–565)] also interacted with TRAF1 and TRAF2 (Fig. 2*A*). Furthermore, a GST-fusion protein with amino acid residues 509–565 of CD30 [CD30(509–565)] alone interacted with TRAF1 and TRAF2 (Fig. 2*A*). These results indicate that there are two independent sites in CD30 for TRAF binding; CD30/TBS-N (CD30/TRAF binding sites-N terminal) and CD30/TBS-C (CD30/TRAF binding sites-C terminal). Further N- and C-terminal deletion analysis within CD30/TBS-C showed that critical amino acid residues for TRAF binding were localized to a patch of 12 amino acid residues (residues 573–584) (Fig. 2*A*).





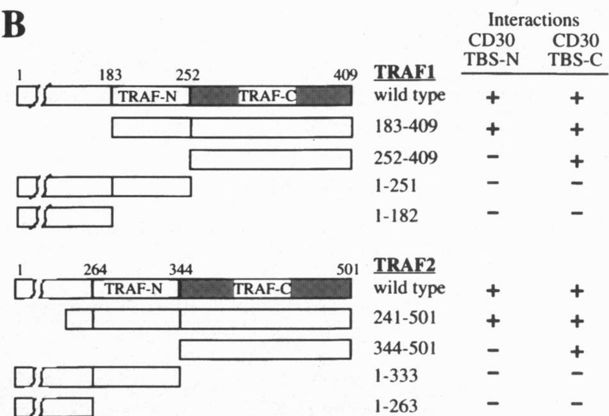


FIG. 2. (*A*) Interaction of CD30 with TRAF. GST-fusion proteins encoding the wild-type or deletion mutants of the CD30 cytoplasmic tail, bound to glutathione beads, were incubated with ^{35}S-Met labeled TRAF1 or TRAF2 translated *in vitro*, and the proteins bound to glutathione beads were analyzed on SDS/PAGE. (*Upper*) Summary of the experiments are presented. The relative amount of TRAF proteins coprecipitated with GST-fusion proteins are as follows: ++, strong binding; +, binding; ±, weak binding; and −, no binding. (*Lower*) Representative experiments of the TRAF–CD30 *in vitro* coprecipitation experiments are shown. *In vitro* translated TRAF1 or TRAF2 was also included on SDS/PAGE (control lane). GST proteins did not interact with TRAF1 or TRAF2 as previously shown (9). (*B*) Interaction of TRAF1 or TRAF2 with CD30. Expression vectors encoding the wild-type or deletion mutants of TRAF1 or TRAF2 fused with the transcription activation domain were cotransformed into yeast with plasmids expressing LexA DNA-binding domain-CD30/TBS-N(residues 509–566) or -CD30/TBS-C(residues 567–595) fusion proteins. Interactions between fusion proteins were scored by measuring β-gal activity of yeast transformants as previously described (20). +, Average β-gal activity of three independent transformants was higher than 1000 Miller units; −, average β-gal activity of three independent transformants was about 50 Miller units. The β-gal activity of negative controls (bait plasmid alone) is about 50 Miller units.

Mapping of TRAF Domains Interacting with CD30/TBS-N and CD30/TBS-C. To determine the region(s) of TRAFs responsible for interaction with CD30/TBS-N and CD30/TBS-C, yeast two-hybrid assays were used with a series of N- or C-terminal TRAF truncation proteins (19). An N-terminal deletion mutant TRAF1 containing only the TRAF domain [TRAF1(183–409)] interacted with both CD30/TBS-N and CD30/TBS-C (Fig. 2*B*). A mutant TRAF1 containing only TRAF-C [TRAF1(252–409)] interacted with CD30/TBS-C but not with CD30/TBS-N. However, a mutant TRAF1 lacking TRAF-C [TRAF1(1–251)] did not interact with either CD30/TBS-N or CD30/TBS-C (Fig. 2*B*). The same results were also true with series of TRAF2 deletion mutants (Fig. 2*B*).

These results suggest that CD30/TBS-N interacts with TRAFs via a complex site containing elements of both TRAF-N and TRAF-C, whereas CD30/TBS-C interacts with TRAFs through TRAF-C (Fig. 2*B*). This suggests that both TRAF-N and TRAF-C contribute to the interaction of CD30 with TRAFs. Therefore, CD30/TRAF interaction seems to be different from the TNF-R2/TRAF and CD40/TRAF interactions, in which only TRAF-C has been shown to play a role (11, 16).

Identification of 5–7 Amino Acid Stretch Critical for TRAF-C Binding. We used *in vitro* coprecipitation assays to determine which amino acid residues of CD30/TBS-C are important for binding to TRAF-C. We changed individual amino acids of CD30/TBS-C and tested the affect of mutations (Fig. 3 and Table 1). Nonconservative changes at amino acid residues Gly-570, Ser-571, Ser-573, Asp-574, Glu-582, Gly-583, or Lys-584 did not affect either TRAF1 or TRAF2 binding. Mutations at residues Val-575, Leu-577, Ser-578, Val-579, or Glu-580 completely inhibited the interaction of GST-fusion proteins with either TRAF1 or TRAF2. Mutations at Met-576 or Glu-581 also abolished the interaction of GST-fusion proteins with TRAF2 but not TRAF1. These results indicate that residues 575–581 of CD30 are critical for interaction with TRAF-C.

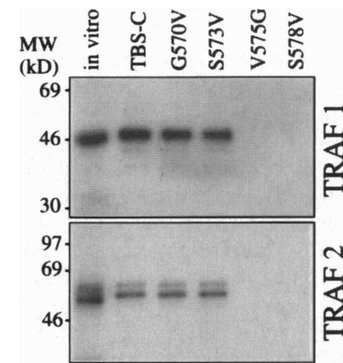


Fig. 3. Mapping of critical amino acid residues of CD30 for TRAF-C binding. GST-fusion proteins with different CD30 mutants were used for *in vitro* coprecipitation assays and representative experiments are shown. Amino acid sequences of the wild-type or mutant CD30 peptides are shown in Table 1. *In vitro* translated TRAF1 or TRAF2 was also included on SDS/PAGE.

DISCUSSION

CD30 has been suggested to play a role in the regulation of lymphocyte activation and differentiation (7, 8). Increased expression of CD30 was also associated with HIV-infected individuals (22) and CD30 cross-linking was shown to induce HIV expression by a NF-κB-dependent pathway in some latently infected T cell lines (21). CD30 has also been shown to regulate cell death (5, 6, 9).

Although it is not well understood how CD30 mediates its pleiotropic signals, we have previously shown that TRAF proteins are associated with the CD30 cytoplasmic tail, suggesting that TRAFs play important roles in CD30-mediated signaling processes (9). Transfection experiments using various C-terminal deletion mutants of CD30 showed that the same C-terminal region of CD30 that interacts with TRAFs is required for CD30-mediated signaling (9).

In this study, we showed that TRAF2 is indeed involved in at least one of the signaling pathways mediated by CD30, NF-κB activation. These results support the idea that TRAF2 is the common mediator for NF-κB activation by TRAF-binding receptors (13). In this study, we also showed that TRAF2 plays an important role in the NF-κB-dependent activation of HIV expression by CD30. Therefore, TRAF2

Table 1. TRAF binding to CD30 peptides

GST-fusion	Sequences	Binding	
		TRAF1	TRAF2
hCD30/TBS-C	GSCSDVMLSVEEEGKEDPLPTAASGK	+	+
mCD30/TBS-C	---E--F-----G----HG--TV-E-	+	+
TBS-C/G570V	V-------------------------	+	+
TBS-C/S571V	-V------------------------	+	+
TBS-C/S573V	---V----------------------	+	+
TBS-C/D574A	----A---------------------	+	+
TBS-C/V575G	-----G--------------------	−	−
TBS-C/M576G	------G-------------------	+	−
TBS-C/L577G	-------G------------------	−	−
TBS-C/S578V	--------V-----------------	−	−
TBS-C/V579G	---------G----------------	−	−
TBS-C/E580A	----------A---------------	−	−
TBS-C/E581A	-----------A--------------	+	−
TBS-C/E582A	------------A-------------	+	+
TBS-C/G583V	-------------V------------	+	+
TBS-C/K584A	--------------A-----------	+	+
TBS-C/E585V	----------------V---------	+	+
TBS-C/D586A	-----------------A--------	+	+

Shown in the table is summary of the TRAF binding to CD30 peptides expressed as GST-fusion proteins, tested by *in vitro* copreciptation experiments as described in Fig. 2*A*. Amino acid residues are represented by single letters. Amino acid residues identical to those of hCD30 TBS-C are indicated by dashes. +, Binding; −, no binding.

hCD30	V	M	L	**S**	V	E	E
mCD30	V	M	F	**S**	V	E	E
hTNF-R2	V	P	F	**S**	K	E	E
mTNF-R2	V	P	F	**S**	Q	E	E
hCD40	V	Q	E	**T**	L	H	G
mCD40	V	Q	E	**T**	L	H	G

FIG. 4. Alignment of the TRAF-C binding sites of CD30 with TNF-R2 and CD40. The 7 amino acid residues of CD30, which were identified to be critical for TRAF-C binding are compared with TNF-R2 and CD40. The 7 amino acids of TNF-R2 and CD40 shown are chosen based on the importance of Ser-342 of TNF-R2, Thr-234 of CD40, and Ser-578 of CD30. Amino acid residues are represented by single letters. Amino acid residues are boxed if they are identical in at least four of the six TRAF binding receptors. The critical Ser or Thr residues identified by mutation analysis are shown in boldface type. Sequences of both murine and human CD30, CD40, and TNF-R2 are previously described (4, 7, 23–26).

may play a central role in the activation of HIV expression by various members of the TNF receptor superfamily.

To understand the interactions of CD30 and TRAFs in more detail, we carried out experiments using the yeast two-hybrid system and coprecipitation of GST-fusion proteins. By expression of various regions of the CD30 cytoplasmic tail as GST-fusion proteins, we were able to demonstrate that CD30 contains two independent TRAF binding sites, CD30/TBS-N and CD30/TBS-C. Furthermore, using the yeast two-hybrid system, we were able to show that CD30/TBS-N binds to a complex site containing elements of both TRAF-N and TRAF-C, whereas CD30/TBS-C binds to TRAF-C.

In addition to its interaction with CD30/TBS-C, TRAF-C also interacts with CD40 and TNF-R2 (10, 11, 13). A previous sequence comparison of the cytoplasmic tails of these receptors did not reveal an obvious region responsible for TRAF binding. Although the TRAF-C domains of TRAF1-3 are highly conserved and share common target proteins (TNF-R2, CD40, or CD30), the relative strengths of interaction with different targets appear to vary (11, 13, 14). Therefore, it is likely that the TRAF-C binding sites are composed of several conserved residues for the minimal epitope and variable residues for the overall affinity and specificity of interaction.

Our study of CD30/TBS-C revealed that GST-fusion proteins with 12 amino acid residues of CD30 were sufficient for TRAF binding. In addition, substitution in a 5–7 amino acid core region of CD30/TBS-C resulted in decreased TRAF binding. It was previously reported that mutation at Thr-234 of CD40 abolished its interaction with TRAF3 (12). Mutation at Ser-578 of CD30 also abolished TRAF-C binding as shown in our study. In addition, mutation at Ser-342 (to Phe) in TNF-R2 significantly decreased the TNF-R2/TRAF interaction (data not shown). These results suggest that Ser or Thr residues play important roles in the interaction of TRAF-C domains with these receptors and allow alignment of putative TRAF-C binding sites. Sequence comparison of the 7 amino acids (residues 575–581) of CD30/TBS-C and the corresponding regions of TNF-R2 and CD40 indicates a highly conserved region (Fig. 4). Although it is tempting to speculate that the conserved amino acid residues shown in Fig. 4 play important roles in the TRAF/receptor interaction, structural delineation of minimum motifs for TRAF-C binding awaits further mutational and x-ray crystallographic studies.

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