CD40−Tumor Necrosis Factor Receptor-Associated Factor (TRAF) Interactions: Regulation of CD40 Signaling through Multiple TRAF Binding Sites and TRAF Hetero-Oligomerization

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ABSTRACT: CD40 is a TNF receptor superfamily member that provides activation signals in antigen-presenting cells such as B cells, macrophages, and dendritic cells. Multimerization of CD40 by its ligand initiates signaling by recruiting TNF receptor-associated factors (TRAFs) to the CD40 cytoplasmic domain. Recombinant human TRAF proteins overexpressed in insect cells were biochemically characterized and used to finely map TRAF binding regions in the human CD40 cytoplasmic domain. TRAF1, TRAF2, TRAF3, and TRAF6, but not TRAF4 or TRAF5, bound directly to the CD40 cytoplasmic domain. CD40 interactions with TRAF2 and TRAF3 were stronger than the interactions with TRAF1 and TRAF6. Full-length TRAF3 and TRAF5 formed hetero-oligomers, presumably through their predicted isoleucine zippers. TRAF3−TRAF5 hetero-oligomers interacted with CD40, indicating that TRAF5 can be indirectly recruited to the CD40 cytoplasmic domain. Overlapping peptides synthesized on cellulose membranes were used to map each TRAF interaction region. TRAF1, TRAF2, and TRAF3 interacted with the same region. The recognition site for TRAF6 was a nonoverlapping membrane proximal region. Using peptides with progressive deletions, a minimal TRAF1, TRAF2, and TRAF3 binding region was mapped to the PVQET sequence in the CD40 cytoplasmic domain. The minimal region for TRAF6 binding was the sequence QEPQEINF. These studies demonstrate that the CD40 cytoplasmic domain contains two nonoverlapping TRAF binding regions and suggest that TRAF1, TRAF2, and TRAF3 could bind competitively to one site. Relative affinities and competition of individual and hetero-oligomeric TRAF proteins for CD40 binding sites may contribute to receptor specificity and cell-type selectivity in CD40-dependent signaling.

Tumor necrosis factor (TNF)[1] receptor superfamily members regulate cellular proliferation, differentiation, and apoptosis in inflammatory and immune responses. Signaling through TNF receptor superfamily members is initiated by oligomerization of the receptors with trimeric ligands, bringing intracellular domains in close proximity. Signal transduction through many of these receptors is mediated in part by a recently identified family of proteins termed TNF receptor-associated factors (TRAFs). Six TRAF family members have been identified (*1*−*8*). Subsets of TRAF proteins have been shown to interact with the TNF receptor family members TNFR2, CD40, CD30, LTβR, ATAR, OX-40, and 4-1BB (*2*−*5*, *8*−*18*). However, the mechanisms mediating receptor specificity and cell-type selectivity of receptor recognition by TRAF proteins and linkages to downstream signal transduction pathways are poorly understood. TRAF1 may be involved in the regulation of apoptosis (*19*). TRAF2, TRAF5, or TRAF6 overexpression in mammalian cells induces nuclear factor κB (NF-κB) and c-Jun N-terminal kinase (JNK) activation (*20*−*24*). TRAF6 additionally mediates extracellular signal-regulated kinase (ERK) activation (*25*). Targeted disruption of the TRAF2 gene in mice results in increased sensitivity to TNF-induced apoptosis and generates severe defects in JNK activation through TNFR1 (*23*). Targeted disruption of the TRAF3 gene in mice causes impaired immune responses to T-dependent antigens and results in early postnatal lethality (*26*).

The conserved C-terminal region of TRAFs (TRAF-C) binds to receptor cytoplasmic domains and mediates interactions with the signaling proteins NF-κB inducing kinase (NIK) and I-TRAF/TANK (*2*, *27*−*29*). A less conserved region N-terminal to the TRAF-C domain (TRAF-N) is a predicted coiled-coil region that mediates TRAF homo- and hetero-oligomerization (*1*−*4*, *30*). With the exception of TRAF1, each TRAF protein contains a predicted N-terminal zinc RING finger and either five or seven predicted zinc fingers. These N-terminal domains are required for TRAF2-mediated NF-κB and JNK activation (*20*−*22*, *30*). When overexpressed, the TRAF-NC domains of TRAF2, TRAF3, TRAF5, and TRAF6 act as dominant negative inhibitors of NF-κB activation (*1*, *8*, *10*, *16*, *31*, *32*). The mechanism of

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[1] Abbreviations: TNF, tumor necrosis factor; TRAF, TNF receptor-associated factor; TNFRII, TNF receptor II; LTβR, lymphotoxin β receptor; ATAR, another TRAF-associated receptor, also termed herpes virus entry mediator (HVEM); NF-κB, nuclear factor κB; JNK, c-Jun N-terminal kinase; NIK, NF-κB inducing kinase; ERK, extracellular signal-regulated kinase; GST, glutathione *S*-transferase; GST−CD40c, GST fusion protein with the CD40 cytoplasmic domain; PCR, polymerase chain reaction.

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inhibition is unclear and could be either competition for binding to receptor cytoplasmic domains or functional inactivation by hetero-oligomerization with full-length TRAF molecules.

The CD40 receptor is expressed constitutively on professional antigen-presenting cells (B lymphocytes, macrophages, dendritic cells), and its expression can be induced with cytokines on fibroblasts, epithelial cells, and endothelial cells (*33*). CD40 signaling in B cells activates multiple signal transduction pathways, including the NF-κB, JNK, and ERK pathways, that result in B cell proliferation and differentiation (*25*, *34*−*37*). Multimerization of CD40 induced by trimeric CD40 ligand initiates signaling, presumably by recruitment and interaction of TRAF proteins with the oligomerized 62 amino acid cytoplasmic domain (*38*). Previous reports have demonstrated that CD40 associates with TRAF2, TRAF3, TRAF5, and TRAF6 (*2*, *4*, *5*, *9*, *10*). Limited deletional and mutational analyses of the CD40 cytoplasmic domain have broadly mapped regions involved in binding of individual TRAF proteins. However, it remains unclear how TRAF proteins may compete or cooperate in mediating signal transduction and how the observed cellular selectivity in CD40 signaling is generated. Fine-structure mapping of TRAF binding sites and a detailed biochemical analysis of the interactions have been limited by low levels of endogenous TRAF proteins.

To gain a detailed understanding of the biochemistry and selectivity of CD40−TRAF interactions, the six human TRAF proteins were expressed in insect cells with recombinant baculoviruses. We demonstrate that TRAF1, TRAF2, TRAF3, and TRAF6 interacted directly with the human CD40 cytoplasmic domain in vitro. Oligomerization studies indicated that only TRAF1 and TRAF2 efficiently hetero-oligomerized through the TRAF domain, but that full-length TRAF3 and TRAF5 interacted, presumably through their predicted isoleucine zippers. Peptide-based mapping analyses precisely defined the binding sites for TRAF1, TRAF2, TRAF3, and TRAF6. TRAF1, TRAF2, and TRAF3 recognized the same amino acid sequence motif whereas TRAF6 interacted with a nonoverlapping membrane proximal region in CD40. These studies provide insight into mechanisms that regulate activation of multiple CD40-dependent signal transduction pathways through TRAF proteins.

MATERIALS AND METHODS

Plasmids and Viruses. To generate the GST fusion protein with the human CD40 cytoplasmic domain, amino acids 216−277 of CD40 (*39*) were PCR-amplified using oligonucleotides 5′-CCCGGGCCATGGCCAAAAAGGTGGCCAAGAAGCCAACC-3′and 5′-CCCGGGAATTCTCATCACTGTCTCTCCTGCACTGAGATGCG-3′and ligated into pCR2.1 (InVitrogen) to generate pCD40c. pCD40c was digested with *Eco*RI, and the 217-bp fragment was ligated into pGEX-4T-3 (Amersham Pharmacia Biotech) to generate pGST−CD40c. Full-length human TRAF1, TRAF2, TRAF3, and TRAF6 were PCR-amplified from a PHA-stimulated human peripheral blood leukocyte cDNA library (Clontech) using oligonucleotide pairs 5′-GGATCCATGGCCTCCAGCTCAGGGAGCAGTCCT-3′ and 5′-GCGGCCGCCTAAGTGCTGGTCTCCACAATGCACTTGA-3′, 5′-AAAAGGAAAAGCGGCCGCTTATTAGAGCCCTGTCAGGTCCA-3′ and 5′-TTGGTTGGATCCTATAAATATGGCTGCAGCTAGCGTGA-3′, 5′-TTGGTTGGATCCTATAAATATGGAGTCGAGTAAAAAGATGGACTC-3′ and 5′-GCGGCCGCTCATCAGGGATCGGGCAGATCCGA-3′, and 5′-GGATCCATGAGTTCTGCTAAACTGTGAAAACAGCTGTGGCTCCAGCCAGTCT-3′ and 5′-GCGGCCGCTATACCCCTGCATCAGTACTTCGTGGGTGA-3′ and ligated into pGem-T (Promega) to make pTRAF1/GemT, pTRAF2/GemT, pTRAF3/GemT, and pTRAF6/GemT, respectively. A phage (HF-2) containing the N-terminus (amino acids 1−59) and plasmid (pHF16-8) encoding the C-terminus (amino acids 18−557) of human TRAF5 were a kind gift from Dr. Hiroyasu Nakano. Using oligonucleotides 5′-CCATGGCTTATTCAGAAGAGCATAAAGGT-3′ and 5′-GTCTGGTGGGGGTTGTGAAGCA-3′ with phage DNA as a template, an N-terminal fragment of TRAF5 was PCR-amplified and ligated into pGem-T to create pN-termTRAF5/GemT. After digestion of pHF16-8 with *Eco*RI, the fragment containing the C-terminus of TRAF5 was ligated into pN-termTRAF5/GemT at *Eco*RI to make pTRAF5/GemT. pTRAF2/GemT, pTRAF3/GemT, and pTRAF6/GemT were digested with *Bam*HI and *Not*I, and the TRAF-containing fragments were ligated into the transplacement vector pVL1393 (InVitrogen) to generate pTRAF2/1393, pTRAF3/1393, and pTRAF6/1393, respectively. An *Nco*I linker (CCCATGGG) (New England Biolabs) was ligated into pVL1393 digested with *Sma*I to create pVL1393/NcoI$^+$. The plasmids pTRAF1/GemT and pTRAF5/GemT were digested with *Nco*I and *Not*I, and the TRAF-containing fragments were ligated into pVL1393/NcoI$^+$ to make pTRAF1/1393 and pTRAF5/1393, respectively.

Oligonucleotides 5′-GATCCGATGGACTACAAAGACGACGATGACAAAGCGTCGGCGTCGGGCATGG-3′ and 5′-AATTCCATGGCGCCCGACGCCGACGCTTTGTCATCGTCGTCTTTGTAGTCCATCG-3′ were annealed and ligated into pVL1393 at the *Bam*HI and *Eco*RI sites to generate pFlag/1393. The TRAF1-NC domain (amino acids 185−416), TRAF2-NC domain (amino acids 272−501), TRAF3-NC domain (amino acids 354−568), TRAF5-NC domain (amino acids 329−557), and TRAF6-NC domain (amino acids 274−511) were PCR-amplified from pTRAF1/GemT, pTRAF2/GemT, pTRAF3/GemT, pTRAF5/GemT, and pTRAF6/GemT using oligonucleotides 5′-CCATGGCCCTGGCTGAGCTGGAGGGGAAGCT-3′, 5′-CCATGGCCTGCGAGAGCCTGGAGAAGAAGACGGCCACTTTTGA-3′, 5′-CCATGGTGGAGTCCCTCCAGAACCGCGTGACCGAGCT-3′, 5′-CCATGGCCTTATTACAAATGGTTAACCAGCAACA-3′, and 5′-CCATGGCCCAGGCTGTTCATAGTTTGAGCGTTATACCCGA-3′ with the respective C-terminal primers mentioned above (except for TRAF5 where the oligonucleotide 5′-GCGGCCGCCTAGAGATCCTCCAGGTCAGT-3′ was used) and ligated into pGem-T to create pTRAF1-NC/GemT, pTRAF2-NC/GemT, pTRAF3-NC/GemT, pTRAF5-NC/GemT, and pTRAF6-NC/GemT, respectively. The *Nco*I to *Not*I TRAF-NC domain-containing fragment from each of these plasmids was ligated into pFlag/1393 at the same sites to make pFlagTRAF1-NC/1393, pFlagTRAF2-NC/1393, pFlagTRAF3-NC/1393, pFlagTRAF5-NC/1393, and pFlagTRAF6-NC/1393, respectively. Full-length and TRAF-NC domain constructs of TRAF1, TRAF2, TRAF3, TRAF5, and TRAF6 were C-terminally tagged with the nine amino acid epitope (SKRSMNDPY) recognized by

the CA21 monoclonal antibody (*40*) by PCR methods to generate TRAF1-CA21, TRAF2-CA21, TRAF3-CA21, TRAF5-CA21, TRAF6-CA21, TRAF1-NC-CA21, TRAF2-NC-CA21, TRAF3-NC-CA21, TRAF5-NC-CA21, and TRAF6-NC-CA21 in pVL1393. Recombinant baculovirus stocks were generated by standard methods (*41*) from the transplacement vectors described above. Viruses Flag-TRAF4-NC and TRAF4-NC-CA21 were described previously (H. G. Miller, H. E. White, and J. J. Crute, unpublished results).

Protein Expression and Purification. Spodoptera frugiperda (Sf21) cells were maintained and infected as described previously (*42*) using medium supplemented with 5% heat-inactivated fetal bovine serum (Hyclone) and 50 μg/mL gentamicin sulfate (Life Technologies, Inc.). All purification procedures were performed at 4 °C. Cytosolic extracts of baculovirus-infected Sf21 cells were prepared as described (*42*), without the addition of ATP or $MgCl_2$, frozen under liquid nitrogen, and stored at −80 °C. Expression of GST and GST−CD40c fusion proteins in *E. coli* strain BL21-(DE3) was by induction with 1.0 mM IPTG for 3 h at 37 °C. Harvested cell paste was resuspended in 2 volumes of lysis buffer (20 mM HEPES, pH 7.5, 200 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 10% v/v glycerol, 1 mM PMSF, 4 μg/mL leupeptin, 4 μg/mL pepstatin A), frozen under liquid nitrogen, and stored at −80 °C. Thawed cell paste was resuspended in an equal volume of lysis buffer, and cells were disrupted by nitrogen cavitation. Extracts were clarified by ultracentrifugation for 75 min at 100000*g* and applied to a glutathione−Sepharose 4B column (Pharmacia Biotech) equilibrated in buffer A (20 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM DTT, 10% v/v glycerol, 0.1 mM EDTA, 0.1 mM EGTA, and 1 mM PMSF). The column was washed with buffer A containing 400 mM NaCl, and protein was eluted with 50% ImmunoPure Gentle Ag/Ab Elution Buffer (Pierce)/50% buffer A containing 400 mM NaCl without DTT. Peak fractions were pooled and chromatographed through Sephacryl S-300 HR (Amersham Pharmacia Biotech) equilibrated in buffer A containing 300 mM NaCl. Purified proteins were quantitated as described (*43*), frozen in aliquots under liquid nitrogen, and stored at −80 °C.

The CA21 cell line producing a mouse IgG1 monoclonal antibody against a peptide epitope (*40*) was a gift from A. Wayne (Boehringer Ingelheim) and was grown and purified as described (*40*, *42*). Purified CA21 antibody was conjugated with *N*-hydroxysuccinimide ester-XX-biotin (Calbiochem) as described (*44*).

GST Coprecipitation Assays. Purified GST−CD40c (20 μg; 590 pmol), Sf21 cytosolic extracts containing CA21 epitope-tagged TRAF, and 10 μL of glutathione−Sepharose 4B were combined in 500 μL of 40 mM HEPES, pH 7.0, 100 mM NaCl, 0.1% Nonidet P-40, 1 mM $MgCl_2$, and 1 mM DTT (binding buffer). Samples were rotated at 4 °C overnight, washed 5 times in ice-cold binding buffer, and eluted in 40 μL of 2% SDS, 50 mM Tris-HCl, pH 6.8, 2% 2-ME (sample buffer). After heating at 95 °C for 5 min, portions of the supernatant were analyzed by SDS−PAGE (12% polyacrylamide Tris−glycine; Novex) followed by staining with Coomassie Brilliant Blue R350 or immunoblotting analysis with anti-Flag or CA21 antibody (see below).

Immunoprecipitation Assays. Immunoprecipitations using Sf21 cytosolic extracts containing CA21 epitope-tagged TRAFs were performed in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) with 20 μL of a 50% slurry of CA21 antibody coupled to Sepharose 4B. Samples were rotated overnight at 4 °C, washed twice in ice-cold RIPA buffer and 3 times in ice cold RIPA buffer containing 500 mM NaCl, and eluted as described above in sample buffer. Portions of the supernatant were analyzed by SDS−PAGE (12% polyacrylamide Tris−glycine; Novex) and either stained with Coomassie Brilliant Blue R350 or transferred to a PVDF membrane (Schleicher & Schuell) by electroblotting. Immunoblot analysis of transferred Flag-tagged proteins was performed by incubating with 0.5 μg/mL anti-Flag BioM2 monoclonal antibody (Kodak) followed by 0.5 μg/mL streptavidin−horseradish peroxidase (Jackson ImmunoResearch) and developed with 3-amino-9-ethyl-carbazole (Sigma) and hydrogen peroxide (0.01%). Antisera specific for TRAF3 and TRAF6 were generated by immunizing rabbits with the peptide CPVFVAQTVLENGTYIKDDTIFIKVIV conjugated to maleimide-activated ovalbumin (Pierce) and peptides CHEKMQRNHLARHLQENTQSH, CDQSEAPVRQNHEEIMDAKPE, and CSTRFCMGSLRREGFQPRSTD conjugated to maleimide-activated keyhole limpet hemocyanin (Pierce), respectively. TRAF2-specific rabbit antiserum was purchased from Santa Cruz Biotechnology (antiserum N-19). Detection of rabbit antisera on PVDF membranes was with horseradish peroxidase conjugated Protein A (BioRad) as described above for the anti-Flag BioM2 antibody.

Peptide Binding Assay. Peptides C-terminally attached to cellulose membranes were synthesized by Jerini BioTools, GmbH (Berlin, Germany). Each peptide spot contained ~10 nmol of peptide/3 mm^2 circle (*45*, *46*). Blocking and binding were performed according to the manufacturer's protocol. Membranes were probed with Sf21 cytosolic extracts containing approximately 1 μg/mL CA21 epitope-tagged TRAF in binding buffer. After electroblotting to PVDF membranes, TRAF proteins were detected with biotin−CA21-epitope specific monoclonal antibody (*40*), streptavidin−horseradish peroxidase (0.5 μg/mL; Jackson ImmunoResearch), and POD chemiluminescence blotting substrate (Boehringer Mannheim). Quantitation was performed by laser densitometry with a Molecular Dynamics Personal Densitometer SI using local averages for background values.

RESULTS

Recombinant TRAF1, TRAF2, TRAF3, and TRAF6 Associate with CD40 in Vitro. Previous studies describing the interactions between CD40 and various TRAF proteins used yeast two-hybrid analysis and coprecipitation assays with extracts from transfected cells or in vitro translated TRAF proteins (*2*, *4*, *5*, *10*). To study direct CD40 interactions with a complete panel of all TRAFs and finely map the TRAF binding sites in the CD40 cytoplasmic domain, recombinant baculoviruses overexpressing each of the six human TRAF proteins were generated. Isolated glutathione *S*-transferase−human CD40 cytoplasmic domain (GST−CD40c) fusion protein was tested for the ability to coprecipitate insect cell-expressed full-length, CA21 epitope-tagged TRAF proteins. TRAF1, TRAF2, TRAF3, and

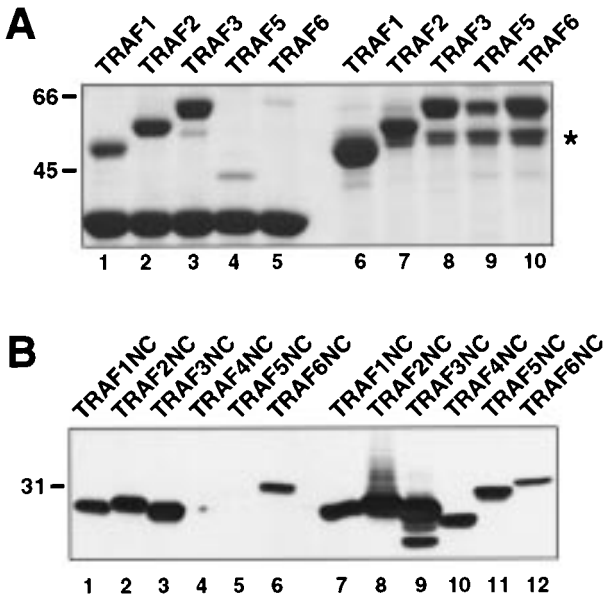


FIGURE 1: In vitro association of TRAF proteins with the CD40 cytoplasmic domain. (A) GST coprecipitation assays and immunoprecipitations were performed as described under Materials and Methods. Lanes 1−5, GST−CD40c coprecipitations using insect cell extracts containing the indicated full-length TRAF-CA21. Lanes 6−10, immunoprecipitations with CA21 monoclonal antibody from the same cell extracts used in the coprecipitations. Precipitated proteins were separated by SDS−PAGE on a 12% polyacrylamide gel and visualized by staining with Coomassie Brilliant Blue. Molecular mass markers listed in kilodaltons. The asterisk indicates the position of immunoglobulin heavy chain from the CA21 antibody. Predicted molecular masses (in kilodaltons) are TRAF1, 47; TRAF2, 57; TRAF3, 66; TRAF5, 66; TRAF6, 61. (B) GST coprecipitation assays were performed as described under Materials and Methods. Lanes 1−6, GST−CD40c coprecipitations using insect cell extracts containing Flag-TRAF-NC domains. Lanes 7−12, 50% of input insect cell extracts used in lanes 1−6 containing Flag-TRAF-NC domain proteins. Proteins were separated by SDS−PAGE on a 12% polyacrylamide gel, electroblotted to a PVDF membrane, and detected using anti-Flag antibody by immunoblot analysis. Predicted molecular masses (in kilodaltons) are Flag-TRAF1-NC, 28; Flag-TRAF2-NC, 28; Flag-TRAF3-NC, 26; Flag-TRAF4-NC, 27; Flag-TRAF5-NC, 28; Flag-TRAF6-NC, 30. Quantitation of the immunoblot in (B) had a smaller dynamic range than quantitation of the Coomassie-stained gel in (A). Relative binding of TRAFs to GST−CD40 is more accurate in (A). Data are representative of the results of two independent experiments.

TRAF6 each coprecipitated with GST−CD40c (Figure 1A, lanes 1−5). The specificity of these interactions was demonstrated by the inability of TRAF proteins to interact with GST alone as detected by immunoblot analysis (data not shown). The interaction of TRAF2, TRAF3, and TRAF6 with CD40 has been described previously (*2*, *4*, *5*, *9*, *10*). An interaction with human TRAF5 was not detected, even after immunoblot analysis (data not shown), although the recombinant TRAF5 was demonstrated to be functional by its interaction with the ATAR cytoplasmic domain (H. G. Miller and J. J. Crute, unpublished results). TRAF4 interacted equally well with GST−CD40c and GST, precluding the ability to assess CD40 interactions with full-length TRAF4 (data not shown). Since an approximately equivalent amount of TRAF was present in each Sf21 cell extract (Figure 1A, lanes 6−10), the relative amount of each TRAF protein coprecipitated by GST−CD40c could be directly compared to give an indication of the relative binding affinity. The majority of the TRAF2 or TRAF3 in the

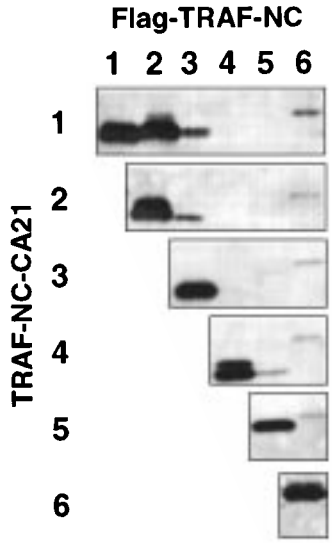


FIGURE 2: TRAF-NC domain-mediated oligomerization. Insect cells were coinfected with all possible pairwise combinations of recombinant baculoviruses expressing Flag (listed on top) and CA21 (listed on the left) epitope-tagged TRAF-NC domains. Numbers designate the TRAF protein number. Immunoprecipitations were performed with insect cell extracts and the CA21 monoclonal antibody; proteins were separated by SDS−PAGE on a 12% polyacrylamide gel, electroblotted to a PVDF membrane, and detected with anti-Flag antibody. Data are representative of the results of two independent experiments.

extracts was coprecipitated by GST−CD40c. However, only a fraction of TRAF1 or TRAF6 coprecipitated with GST−CD40c. Although it appeared that more TRAF1 bound to GST−CD40 than TRAF6, TRAF1 expression in insect cell extracts was high relative to the other TRAFs, and the relative strength of TRAF1 and TRAF6 interactions with CD40 could not be determined. These results indicate that the TRAF2 and TRAF3 interactions with CD40 were considerably stronger than the TRAF1 and TRAF6 interactions.

The specificity of the TRAF-CD40 interactions was confirmed by testing the ability of GST−CD40c to precipitate the TRAF-NC domain of each TRAF. Consistent with the results using full-length TRAF proteins, the TRAF-NC domains of human TRAF1, TRAF2, TRAF3, and TRAF6 interacted with GST−CD40c (Figure 1B, lanes 1−6). TRAF4-NC domain and TRAF5-NC domain did not interact with CD40. None of the TRAF-NC domains coprecipitated with GST (data not shown).

Hetero-oligomerization of TRAF Proteins. TRAF1, TRAF2, TRAF3, and TRAF6 have been demonstrated to homo-oligomerize through the TRAF domain (*1*−*4*). TRAF1 and TRAF2 hetero-oligomerize (*3*, *30*); however, the ability of other TRAFs to hetero-oligomerize has not been systematically addressed. Since hetero-oligomerization of TRAFs could result in activation of additional signaling pathways through CD40, the ability of all pairwise combinations of TRAFs to hetero-oligomerize was examined. Immunoprecipitations with CA21 antibody were performed using extracts from insect cells coinfected with two recombinant baculoviruses, one expressing a CA21-tagged TRAF domain and the other expressing a Flag-tagged TRAF domain. Associated TRAFs were detected with anti-Flag antibody. Each TRAF domain homo-oligomerized, and TRAF1-NC and TRAF2-NC hetero-oligomerized as efficiently as each homo-oligomerized (Figure 2). This confirms and extends

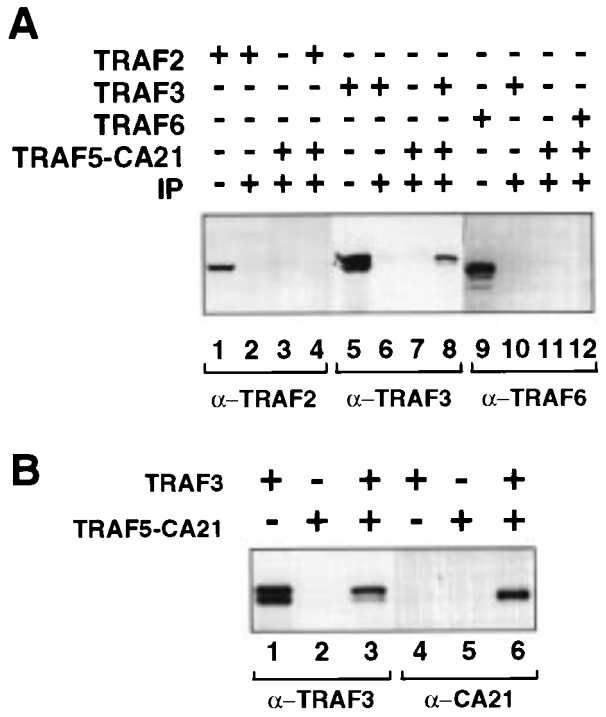


FIGURE 3: Hetero-oligomerization of TRAF3 and TRAF5. (A) Insect cells were infected with various combinations of recombinant baculoviruses expressing TRAF5-CA21, TRAF2, TRAF3, or TRAF6 as indicated by "+" over the lanes. Proteins from whole cell extracts (IP, indicated by "−") or immunoprecipitations of TRAF5-CA21 using the CA21 monoclonal antibody (IP, indicated by "+") were separated by SDS−PAGE on a 12% polyacrylamide gel, electroblotted to a PVDF membrane, and detected with antisera specific for TRAF2 (lanes 1−4), TRAF3 (lanes 5−8), or TRAF6 (lanes 9−12). The internal controls on the immunoblots demonstrate the specificity of the antisera to TRAF2, TRAF3, and TRAF6. (B) Insect cell extracts in lanes 6−8 in panel A were used for GST−CD40 coprecipitation assays as described under Materials and Methods. Precipitated proteins were separated by SDS−PAGE on a 12% polyacrylamide gel, electroblotted to a PVDF membrane, and detected using anti-TRAF3 antisera (lanes 1−3) or CA21 antibody (lanes 4−6). Data are representative of the results of two independent experiments.

previous reports (*3*, *30*). TRAF1-NC and TRAF2-NC each hetero-oligomerized with TRAF3-NC to a lesser extent. Weaker interactions were detected between TRAF4-NC or TRAF6-NC and each of the other TRAFs (Figure 2). This indicates that TRAF hetero-oligomers do not form efficiently through the TRAF domains of TRAF3, TRAF4, TRAF5, and TRAF6.

TRAF3 and TRAF5 are the only TRAF proteins that contain a predicted isoleucine zipper N-terminal to the TRAF domain. Since isoleucine zipper sequences have been shown to mediate protein homo-oligomerization (*47*), it was possible that full-length TRAF3 and TRAF5 might interact through the isoleucine zipper. To test this, insect cells were infected with individual baculoviruses or with pairwise combinations of baculoviruses expressing TRAF5-CA21 and either TRAF2, TRAF3, or TRAF6. Only full-length TRAF3 and TRAF5 coimmunoprecipitated (Figure 3A, lane 8). Since the TRAF domains of TRAF3 and TRAF5 did not interact (Figure 2), and TRAFs that lack the predicted isoleucine zipper (TRAF2 and TRAF6) did not interact with TRAF5, this indicates that the TRAF3−TRAF5 interaction is most likely mediated through the isoleucine zipper domains. Additionally, the TRAF3−TRAF5 hetero-oligomer coprecipitated with GST−CD40c (Figure 3B, lane 6). Thus, although human TRAF5 did not interact directly with the CD40 cytoplasmic domain, TRAF5 was capable of being recruited indirectly to CD40 as a hetero-oligomer with TRAF3.

Mapping the TRAF Binding Sites on CD40. To map the TRAF1, TRAF2, TRAF3, and TRAF6 binding sites on the CD40 cytoplasmic domain, a linear peptide binding approach was employed. Overlapping peptides of 14 and 10 amino acid residues scanning across the CD40 cytoplasmic domain (with an overlap of 13 and 9 residues, respectively) were synthesized on cellulose membranes (Figure 4 A,B). Insect cell extracts containing individual TRAF proteins were tested for binding to the immobilized peptides. TRAF1 bound to peptides in two separate regions of the CD40 cytoplasmic domain (Figure 4C,D). Binding to the membrane distal region (Figure 4C, peptides 30−34) correlated with peptides containing the sequence PVQET that has been implicated as being important for TRAF2 and TRAF3 binding to CD40 (*11*, *27*). Optimal TRAF1 binding in this region occurred when the PVQET sequence was positioned toward the N-terminus of the peptides. TRAF1 interaction with a membrane proximal region (Figure 4C, peptides 1−5) correlated with the presence of positively charged residues in the peptides (see Figure 4A,B). Similar interactions with this region were observed for TRAF2 and TRAF3 when 20-mer peptides were used (data not shown). This binding to the membrane proximal region may be due to nonspecific ionic interactions of the TRAFs with the numerous positively charged residues in these peptides (Figure 4A).

TRAF2 and TRAF3 bound strongest to a single region defined by the 14 and 10 amino acid residue peptides (Figure 4C,D). TRAF2 and TRAF3 bound the best to the same 10 amino acid peptides with the PVQET sequence at the C-terminus of the peptide (Figure 4D, peptides 30, 31). Binding decreased as the PVQET sequence was positioned toward the N-terminus of the peptide. For TRAF3, low-level binding to 14-mers was also detected when a similar sequence (PVTQE) was positioned near the C-terminus of the peptides (Figure 4A,C, peptides 36, 37). TRAF2 and TRAF3 binding to the PVTQE sequence was stronger when 20-mer peptides were used. An increased binding was observed with PVTQE in conjunction with the PVQET sequence (data not shown). TRAF6 interacted with a series of 14 amino acid residue peptides corresponding to a region N-terminal to the PVQET sequence (Figure 4C, peptides 10, 12−16). A single 10 amino acid peptide interacted weakly with TRAF6 (Figure 4D, peptide 14). This TRAF6 binding sequence was positioned within a larger region previously identified as required for TRAF6 binding to CD40 (*10*).

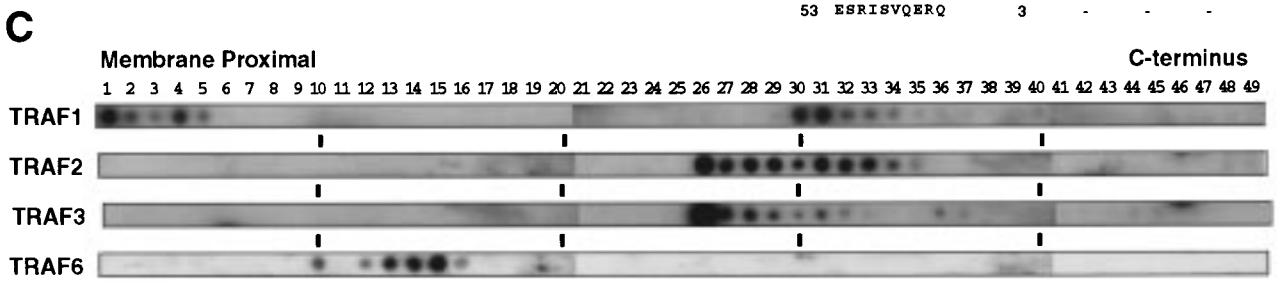
Defining Minimal TRAF Binding Sites in CD40. To more finely map the TRAF1, TRAF2, TRAF3, and TRAF6 binding sites in CD40, peptides containing the TRAF1, TRAF2, TRAF3, or TRAF6 binding regions were synthesized on cellulose membranes with progressive single amino acid deletions from either the N-terminus, the C-terminus, or both termini. Progressive removal of residues from the C-terminus of a 14 amino acid long TRAF1 binding region peptide increased TRAF1 binding. Removal of Thr^{254} nearly abolished binding (Figure 5A, peptides 1−12). When amino acids were deleted from the N-terminus, TRAF1 binding was

A

	14-mer peptides	Relative Binding TRAF1	TRAF2	TRAF3	TRAF6
1	KKVAKKPTNKAPHP	100	-	-	-
2	KVAKKPTNKAPHPK	23	-	-	-
3	VAKKPTNKAPHPKQ	11	-	-	-
4	AKKPTNKAPHPKQE	59	-	-	-
5	KKPTNKAPHPKQEP	11	-	-	-
6	KPTNKAPHPKQEPQ	-	-	-	-
7	PTNKAPHPKQEPQE	-	-	-	-
8	TNKAPHPKQEPQEI	-	-	-	-
9	NKAPHPKQEPQEIN	-	-	-	-
10	KAPHPKQEPQEINF	-	-	-	23
11	APHPKQEPQEINFP	-	-	-	-
12	PHPKQEPQEINFPD	-	-	-	11
13	HPKQEPQEINFPDD	-	-	-	41
14	PKQEPQEINFPDDL	-	-	-	64
15	KQEPQEINFPDDLP	-	-	-	100
16	QEPQEINFPDDLPG	-	-	-	5
17	EPQEINFPDDLPGS	-	-	-	-
18	PQEINFPDDLPGSN	-	-	-	-
19	QEINFPDDLPGSNT	-	-	-	-
20	EINFPDDLPGSNTA	-	-	-	-
21	INFPDDLPGSNTAA	-	-	-	-
22	NFPDDLPGSNTAAP	-	-	-	-
23	FPDDLPGSNTAAPV	-	-	-	-
24	PDDLPGSNTAAPVQ	-	-	-	-
25	DDLPGSNTAAPVQE	-	-	-	-
26	DLPGSNTAAPVQET	-	100	100	-
27	LPGSNTAAPVQETL	-	35	9	-
28	PGSNTAAPVQETLH	-	54	13	-
29	GSNTAAPVQETLHG	-	61	9	-
30	SNTAAPVQETLHGC	26	28	5	-
31	NTAAPVQETLHGCQ	42	72	13	-
32	TAAPVQETLHGCQP	10	54	7	-
33	AAPVQETLHGCQPV	12	54	-	-
34	APVQETLHGCQPVT	10	20	-	-
35	PVQETLHGCQPVTQ	4	7	-	-
36	VQETLHGCQPVTQE	-	-	12	-
37	QETLHGCQPVTQED	-	-	5	-
38	ETLHGCQPVTQEDG	-	-	-	-
39	TLHGCQPVTQEDGK	-	-	-	-
40	LHGCQPVTQEDGKE	-	-	-	-
41	HGCQPVTQEDGKES	-	-	-	-
42	GCQPVTQEDGKESR	-	-	-	-
43	CQPVTQEDGKESRI	-	-	-	-
44	QPVTQEDGKESRIS	-	-	-	-
45	PVTQEDGKESRISV	-	-	-	-
46	VTQEDGKESRISVQ	-	-	-	-
47	TQEDGKESRISVQE	-	-	-	-
48	QEDGKESRISVQER	-	-	-	-
49	EDGKESRISVQERQ	-	-	-	-

B

	10-mer peptides	Relative Binding TRAF1	TRAF2	TRAF3	TRAF6
1	KKVAKKPTNK	100	-	-	-
2	KVAKKPTNKA	71	-	-	-
3	VAKKPTNKAP	15	-	-	-
4	AKKPTNKAPH	-	-	-	-
5	KKPTNKAPHP	4	-	-	-
6	KPTNKAPHPK	-	-	-	-
7	PTNKAPHPKQ	-	-	-	-
8	TNKAPHPKQE	-	-	-	-
9	NKAPHPKQEP	-	-	-	-
10	KAPHPKQEPQ	-	-	-	-
11	APHPKQEPQE	-	-	-	-
12	PHPKQEPQEI	-	-	-	-
13	HPKQEPQEIN	-	-	-	-
14	PKQEPQEINF	-	-	-	100
15	KQEPQEINFP	-	-	-	-
16	QEPQEINFPD	-	-	-	-
17	EPQEINFPDD	-	-	-	-
18	PQEINFPDDL	-	-	-	-
19	QEINFPDDLP	-	-	-	-
20	EINFPDDLPG	-	-	-	-
21	INFPDDLPGS	-	-	-	-
22	NFPDDLPGSN	-	-	-	-
23	FPDDLPGSNT	-	-	-	-
24	PDDLPGSNTA	-	-	-	-
25	DDLPGSNTAA	-	-	-	-
26	DLPGSNTAAP	-	-	-	-
27	LPGSNTAAPV	-	-	-	-
28	PGSNTAAPVQ	-	-	-	-
29	GSNTAAPVQE	-	-	-	-
30	SNTAAPVQET	5	90	95	-
31	NTAAPVQETL	16	100	100	-
32	TAAPVQETLH	7	70	42	-
33	AAPVQETLHG	-	96	46	-
34	APVQETLHGC	26	20	17	-
35	PVQETLHGCQ	44	-	6	-
36	VQETLHGCQP	3	-	-	-
37	QETLHGCQPV	-	-	-	-
38	ETLHGCQPVT	-	-	-	-
39	TLHGCQPVTQ	-	-	-	-
40	LHGCQPVTQE	-	-	-	-
41	HGCQPVTQED	-	-	-	-
42	GCQPVTQEDG	-	-	-	-
43	CQPVTQEDGK	-	-	-	-
44	QPVTQEDGKE	-	-	-	-
45	PVTQEDGKES	-	-	-	-
46	VTQEDGKESR	-	-	-	-
47	TQEDGKESRI	-	-	-	-
48	QEDGKESRIS	-	-	-	-
49	EDGKESRISV	-	-	-	-
50	DGKESRISVQ	-	-	-	-
51	GKESRISVQE	3	-	-	-
52	KESRISVQER	14	5	-	-
53	ESRISVQERQ	3	-	-	-



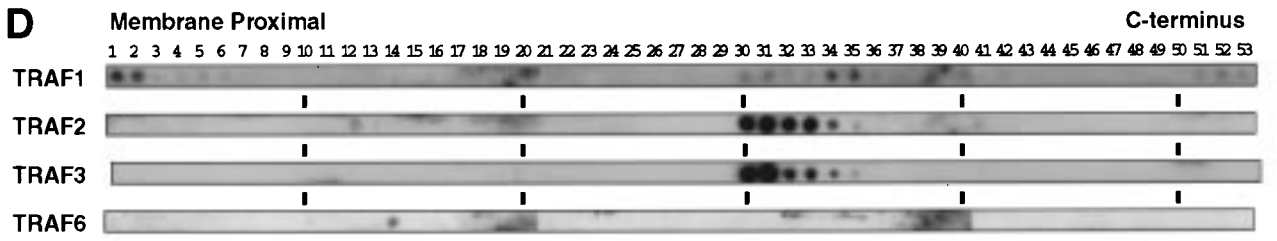


FIGURE 4: TRAF protein binding to peptides scanning the CD40 cytoplasmic domain. Overlapping 14 amino acid residue (A) and 10 amino acid residue (B) peptides synthesized in spots on cellulose membranes were probed with TRAF1, TRAF2, TRAF3, or TRAF6 as described under Materials and Methods. TRAF proteins bound to 14-mer (C) and 10-mer (D) peptides were visualized by chemiluminescent detection and are shown as scans of the exposed films. Peptide spot numbers correspond to the peptide sequences in panels A and B. Relative levels of binding in (C) and (D) were quantitated and the relative integrated areas indicated in (A) and (B). Numbers were normalized within each binding assay to give the darkest spot a value of 100. Relative binding below 3% is indicated by a dash (−). Data are representative of the results of two independent experiments for TRAF1 and TRAF6 and four independent experiments for TRAF2 and TRAF3.

eliminated once Pro^{250} was removed (Figure 5A, peptides 13−24). When amino acids were deleted simultaneously from both ends of the peptide, TRAF1 bound weakly to the sequence VQETLHGC (Figure 5A, peptides 25−29). This

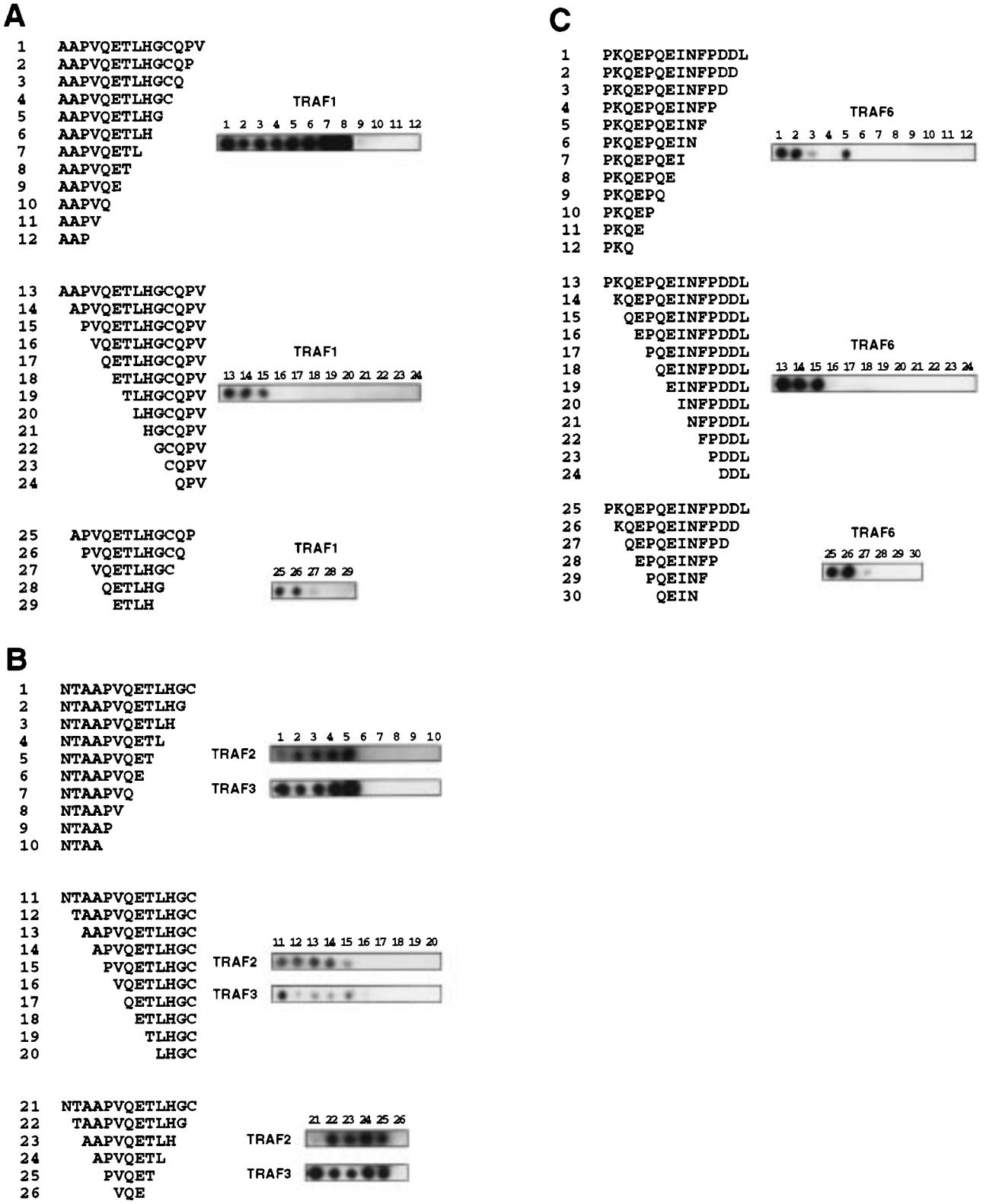


FIGURE 5: TRAF1, TRAF2, TRAF3, and TRAF6 protein binding to peptides with progressive deletions. Based on the TRAF binding peptides in Figure 4, peptides with progressive deletions from the C-terminus, N-terminus, or both termini were synthesized on cellulose membranes and probed for TRAF1 (A), TRAF2 or TRAF3 (B), or TRAF6 (C) binding as in Figure 4. TRAF proteins bound to the membranes were visualized by chemiluminescent detection, were scanned, and are shown to the right of each set of peptides. Numbers indicate the peptide sequence corresponding to individual peptide spots. Peptide strips that were bound to TRAF2 in (B) containing peptides 1−10 and 21−26 were exposed for shorter times relative to the peptide strip containing peptides 11−20. Although peptides 1 and 21 appear less positive than peptide 11, the TRAF2 binding on equal exposures was equivalent for peptides 1, 11, and 21.

indicates that the sequence PVQET is required for optimal TRAF1 binding to CD40, but that Pro^{250} and Thr^{254} may not be essential.

Progressive removal of amino acids from the C-terminus of a 13 amino acid long TRAF2 and TRAF3 binding region peptide increased the ability of TRAF2 or TRAF3 to bind.

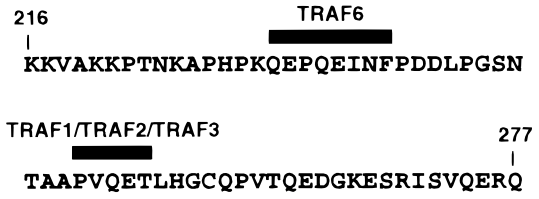


FIGURE 6: Minimal TRAF binding sites in the CD40 cytoplasmic domain. The minimal binding site for each TRAF protein is indicated by a solid bar over the human CD40 cytoplasmic domain sequence.

When Thr^{254} was deleted, TRAF2 and TRAF3 binding to the peptide was eliminated (Figure 5B, peptides 1−10). Deletions from the N-terminus had no effect on TRAF2 and TRAF3 interactions until Pro^{250} was removed. The deletion of Pro^{250} reduced TRAF3 binding and abolished TRAF2 binding (Figure 5B, peptides 11−20). TRAF2 and TRAF3 did not bind to peptides once Val^{251} was removed. Simultaneous deletion of residues from both ends of the peptide demonstrated that the sequence PVQET was sufficient for nearly optimal levels of TRAF2 and TRAF3 binding (Figure 5B, peptide 25).

The ability of TRAF6 to bind to a 14 amino acid long peptide was eliminated once Phe^{238} was removed by progressive C-terminal deletions (Figure 5C, peptides 1−12). Removal of residues from the N-terminus had no effect until Gln^{231} was removed. The deletion of Gln^{231} nearly eliminated TRAF6 binding (Figure 5C, peptides 13−24). Simultaneous deletions from both ends of the peptide supported the importance of Gln^{231} for optimal binding (Figure 5, peptides 25−30). The sequence QEPQEINF may be a core TRAF6 binding sequence; however, adjacent residues appeared to contribute to optimal TRAF6 binding. Together with the scanning results in Figure 4, the peptides with progressive amino acid deletions define the minimal TRAF1, TRAF2, TRAF3, and TRAF6 binding regions in the CD40 cytoplasmic domain (summarized in Figure 6).

DISCUSSION

A subset of TNF receptor family members including CD40, TNFR2, CD30, LTβR, ATAR, OX40, and 4-1BB signal by directly interacting with TRAF proteins following receptor cross-linking. Previous studies have addressed the interactions of individual TRAFs with subsets of TNF receptors, but no complete and comparative biochemical analysis of TRAF interactions with CD40 has been performed. By testing each of the six recombinant TRAF proteins for interaction with the CD40 cytoplasmic domain, we found that, consistent with previous studies, CD40 interacted with TRAF2, TRAF3, and TRAF6 (*2*, *4*, *5*, *10*). Additionally, a direct TRAF1 interaction with CD40 was demonstrated. A hierarchy of CD40−TRAF interaction could be proposed from coprecipitation experiments. CD40 interactions with TRAF2 and TRAF3 were considerably stronger than interactions with TRAF1 and TRAF6. Thus, the inability to detect an interaction between TRAF1 and CD40 in earlier reports (*11*, *31*) may be due to a significantly lower affinity of TRAF1 for CD40 when compared to TRAF2 and TRAF3. In support of our findings, transiently expressed TRAF1 can be coimmunoprecipitated with CD40 in a CD40 ligand-stimulated B cell line (R. J. Noelle, personal communication). Two studies have shown conflicting results on the ability of TRAF5 to bind CD40 (*8*, *9*). Although these studies used mouse TRAF5 and CD40, our results using human proteins consistently show no direct binding of TRAF5 to CD40. Additionally, no evidence for direct binding of TRAF4 to CD40 was obtained.

Homo-oligomerization of TRAF1, TRAF2, TRAF3, and TRAF6 has been reported to be mediated by the TRAF domain (*1*−*4*, *30*). We confirmed that the TRAF domains of TRAF1, TRAF2, TRAF3, and TRAF6 homo-oligomerize and have extended these results to demonstrate homo-oligomerization of TRAF4 and TRAF5. TRAF1 is recruited to the TNFR2 cytoplasmic domain indirectly by hetero-oligomerizing with TRAF2 (*3*, *30*). This TRAF domain-mediated hetero-oligomerization allows for indirect recruitment of TRAF proteins to receptors. In coexpression experiments in insect cells, we found that only TRAF1 and TRAF2 hetero-oligomerized efficiently through the TRAF domain. TRAF1 and TRAF2 each interacted weakly with TRAF3, but the physiological significance of this interaction is unclear considering the weakness of the interaction and the low endogenous levels of TRAF proteins in cells. No other TRAF domain hetero-oligomers formed to an appreciable extent. The limited ability of TRAFs to hetero-oligomerize through the TRAF domain restricts the potential combinations of TRAFs recruited to receptors. Thus, extensive hetero-oligomerization of TRAFs would not be expected to play a role in the complexity of TNF receptor superfamily signaling.

Despite an inability to hetero-oligomerize through the TRAF domain, full-length TRAF3 and TRAF5 interacted (Figure 3). TRAF3 and TRAF5 TRAF domains did not interact, and full-length TRAF5 did not interact with full-length TRAF2 or TRAF6 that have similar zinc RING and zinc finger motifs. This suggests that the predicted isoleucine zipper regions in TRAF3 and TRAF5 may mediate their interaction. It is possible that TRAF3 and TRAF5 may form higher order homo- and hetero-oligomers when compared with oligomers formed through the TRAF domain alone. Consistent with this possibility, TRAF3/TRAF5 hetero-oligomers were found to coprecipitate with GST−CD40c. Consequently, TRAF5 could be recruited to CD40 and other receptors through interactions with TRAF3. These findings suggest that TNF receptors which interact with TRAF3 would be capable of indirectly recruiting TRAF5. Additionally, the mechanism by which overexpressed TRAF3 acts as a negative modulator of NF-κB activation (*16*) could be a down-modulation of TRAF5 recruitment to receptors.

Obtaining cell-type selectivity in activating CD40-dependent signal transduction pathways (*37*) through TRAFs has been difficult to understand because all TRAFs except TRAF6 appear to recognize the same site on CD40. It is not clear whether TRAFs compete for CD40 binding in cells. By fine structure peptide-based mapping, the sequence PVQET was demonstrated to be sufficient for optimal binding of TRAF1, TRAF2, and TRAF3. The accuracy of mapping TRAF binding sites using peptides covalently linked to membranes has been supported by preliminary studies with soluble peptides. The ability of soluble versions of the peptides in Figure 5 to compete for CD40−TRAF2 and CD40−TRAF3 binding correlated well with TRAF2 and TRAF3 binding to the cellulose-linked peptides (M. R. Kehry and J. J. Crute, unpublished results). Since the binding

affinity of TRAF2 and TRAF3 to CD40 in coprecipitation experiments appeared similar, it is thus likely that, depending on the relative concentrations of TRAF2 and TRAF3 in the cell, these two TRAFs could compete for binding to CD40. This additionally may account for some of the similar cellular effects of dominant-negative TRAF2 and TRAF3 proteins. Interestingly, a 30-fold decrease in the density of peptides coupled to the cellulose membranes dramatically weakened, although did not eliminate, the signal for TRAF3 binding but left TRAF2 binding intact (S. S. Pullen and M. R. Kehry, unpublished results). Thus, the extent of receptor cross-linking might also contribute to a hierarchy of TRAF binding in activated cells. A binding hierarchy of TRAFs to CD40, combined with possible differential levels of TRAF expression in different cell types, suggests that some cellular selectivity in CD40-dependent signaling (*37*) could be established by competition of TRAF1, TRAF2, and TRAF3 for binding to cross-linked receptors.

The interaction of TRAF1 with the PVQET sequence was weaker when compared with interactions of TRAF2 and TRAF3 (Figure 4C,D). Sequences similar to the PVQET sequence of CD40 that support binding of TRAF1, TRAF2, and TRAF3 are found in LMP1 (PQQAT), CD30 (PEQET), and OX40 (PIQEE) (*11*, *13*, *17*). Our finding that TRAF1 binds to CD40 is therefore not surprising. Interestingly, the TRAF binding motifs in LMP1, CD30, and CD40 [previously designated PXQXT/S (*48*)] have different hierarchies of affinity for TRAF1, TRAF2, and TRAF3 (*11*, *17*). For example, TRAF3 interacts strongest with the LMP1 sequence, and TRAF2 interacts the weakest with LMP1 (*17*). Therefore, sequences flanking PXQXT/S or the amino acids at the second and fourth positions in the motif may increase or decrease the relative affinities of TRAF1, TRAF2, and TRAF3 binding. Thus, a distinct hierarchy of affinities of TRAF1, TRAF2, and TRAF3 for the PXQXT/S motif in different receptors may alter the outcome of receptor cross-linking to provide receptor specificity in signal transduction. We are currently testing this idea using the CD40 and OX40 cytoplasmic domains.

TRAF2 and TRAF3 also interacted with a membrane distal region distinct from the PVQET sequence. Peptides 14 amino acid residues in length containing the sequence PVTQE interacted weakly with TRAF3, but not with TRAF2. However, 20 amino acid residue peptides containing the PVTQE sequence interacted well with both TRAF2 and TRAF3. This may indicate that CD40 contains two distinct TRAF2 and TRAF3 binding sites. This possibiltiy is analogous to the two binding sites for TRAF1 and TRAF2 in the CD30 cytoplasmic domain (*11*, *13*). Alternatively, CD40 may possess two separate regions that form contacts with the same TRAF subunit.

In contrast to TRAF1, TRAF2, and TRAF3, TRAF6 interacted with a distinct nonoverlapping sequence in the CD40 cytoplasmic domain (amino acids 231−238; Figure 6) that is contained within a larger region previously defined as a TRAF6 binding site (*10*). It is thus possible that multimerized CD40 may be able to interact simultaneously with TRAF6 and either TRAF1, TRAF2, or TRAF3. Additionally, the TRAF6 binding region in CD40 shares limited sequence similarity with the TRAF1, TRAF2, and TRAF3 interaction site (Figure 6), although similar amino acids are present (P, Q, and E). CD40 is the only TNF receptor family member demonstrated to directly interact with TRAF6, which may impart unique signaling outcomes to this receptor.

After multimerization of TNF receptor family members by their respective trimeric ligands, the initial event in signaling is thought to be mediated by a transient recruitment of TRAF proteins (*38*). Since there is a report of constitutive association of TRAF2 and CD40 prior to CD40 cross-linking (*49*), it is possible that some stable CD40−TRAF interactions could form during coprecipitation assays. Preliminary biochemical studies on a monomeric form of the 62 amino acid CD40 cytoplasmic domain suggest that it is unstructured and is a poor competitor of GST−CD40c−TRAF interactions in coprecipitation assays (S. S. Pullen and J. J. Crute, unpublished results). These data suggest that the non-cross-linked CD40 receptor on unstimulated cells would be a poor binding site for TRAFs. These studies support a model where the cytoplasmic domains of multimerized CD40 form binding sites with differing affinities for individual TRAFs depending upon the extent of CD40 cross-linking. Additionally, the relative in vivo concentrations of different TRAF proteins in different cell types would establish a dynamic equilibrium of TRAF binding and an overall integrated signaling response. Defining differences in the downstream signaling capacities of TRAF2, TRAF5, and TRAF6 and the outcomes of CD40 interactions with TRAF1 and TRAF3 remain to be determined. Although it is thought that TRAFs are released from CD40 shortly after initiation of signaling (*38*, *49*), the mechanism of this release, the involvement of protein kinases, and molecular links to downstream signaling pathways require further elucidation.

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