Tumor necrosis factor receptor-associated factors (TRAFs) associate with the CD40 cytoplasmic domain and initiate signaling after CD40 receptor multimerization by its ligand. We used saturating peptide-based mutational analyses of the TRAF1/TRAF2/TRAF3 and TRAF6 binding sequences in CD40 to finely map residues involved in CD40-TRAF interactions. The core binding site for TRAF1, TRAF2, and TRAF3 in CD40 could be minimally substituted. The TRAF6 binding site demonstrated more amino acid sequence flexibility and could be optimized. Point mutations that eliminated or enhanced binding of TRAFs to one or both sites were made in CD40 and tested in quantitative CD40-TRAF binding assays. Sequences flanking the core TRAF binding sites were found to modulate TRAF binding, and the two TRAF binding sites were not independent. Cloned stable transfectants of human embryonic kidney 293 cells that expressed wild type CD40 or individual CD40 mutations were used to demonstrate that both TRAF binding sites were required for optimal NF-κB and c-Jun N-terminal kinase activation. In contrast, p38 mitogen-activated protein kinase activation was primarily dependent upon TRAF6 binding. These studies suggest a role in CD40 signaling for competitive TRAF binding and imply that CD40 responses reflect an integration of signals from individual TRAFs.

CD40 is a tumor necrosis factor (TNF) receptor superfamily member that provides activation signals in antigen-presenting cells such as B cells, macrophages, and dendritic cells (1). CD40 signaling is initiated by receptor oligomerization upon binding the trimeric ligand CD154 (CD40 ligand/gp39). Numerous and different outcomes of CD40 signaling occur in distinct cell types and imply a complex regulation of CD40 signal transduction. Several different signal transduction pathways have been demonstrated to be activated following CD40 oligomerization. CD40-mediated signaling results in NF-κB activation (2, 3), c-Jun N-terminal kinase (JNK) activation (4–7), and p38 mitogen-activated protein kinase (MAPK) activation (8, 9). Additionally, signaling through CD40 mediates activation of protein-tyrosine kinases and phosphatases with an effect on protein kinase C remaining controversial (reviewed in Ref. 10). The CD40 cytoplasmic domain binds directly to several TNF receptor-associated factors (TRAFs), and this interaction is thought to initiate CD40 signaling (11–15). TRAF interactions with CD40 appear to require an oligomerized receptor cytoplasmic domain and are thought to be responsible for initiating activation of most of the CD40-mediated signaling pathways. The signaling functions of most of the six TRAF family members have been characterized genetically and biochemically. A function for TRAF1 in regulating apoptosis has been suggested from transgenic mice expressing a dominant negative TRAF1 transgene (16). Studies using TRAF2 knockout mice and transgenic mice expressing a dominant negative form of TRAF2 showed that TRAF2 is required for JNK activation but has limited involvement in NF-κB activation (17, 18). TRAF2 also mediates p38 MAPK activation when transiently overexpressed (19). Although the molecular function of TRAF3 is unclear, mice irradiated and bone marrow reconstituted with fetal liver cells from TRAF3 knockout animals exhibited impaired T cell priming (20). The outcome of TRAF4 signaling is unknown. In transient overexpression studies, TRAF5 and TRAF6 mediated NF-κB and JNK activation (15, 21–25). Only TRAF6 has been demonstrated to activate extracellular signal-regulated kinase (21).

The cytoplasmic domain of human CD40 interacts directly with TRAF1, TRAF2, TRAF3, and TRAF6 (11–15). In previous studies, the consensus sequence PXQ(T/S) (26) has been designated as a TRAF1, TRAF2, and TRAF3 binding site. In the CD40 cytoplasmic domain, this sequence is ~PVPQET. Mutations and deletions have been made in the ~PVPQET sequence of CD40 that eliminate TRAF2 and TRAF3 binding (14, 27). However, specific mutations in the CD40 cytoplasmic domain that individually affect TRAF1, TRAF2, or TRAF3 binding have not been identified. Although human TRAF5 does not interact directly with the CD40 cytoplasmic domain, it can be recruited indirectly as a hetero-oligomer with TRAF3 (11). The TRAF6 binding site has been mapped to a membrane-proximal region (~QEPEINF) (11, 15). However, it is not known which of these amino acid residues in CD40 are critical for TRAF6 interaction. Whether the two TRAF binding regions in the CD40 cytoplasmic domain are independent and can simultaneously mediate binding of different TRAFs or whether binding of a TRAF to one binding region would block binding of a
second TRAF has not been determined. Additionally, the role of individual TRAFs in activating different CD40-dependent signaling pathways has not been defined.

To gain a better understanding of the interactions between TRAFs and their cognate binding sites on the CD40 cytoplasmic domain and define the role of individual TRAFs in CD40 signaling, saturating peptide-based mutational analyses of the TRAF binding sites in the CD40 cytoplasmic domain were performed. TRAF1, TRAF2, and TRAF3 had similar binding specificities for the core 250PVQET sequence with few amino acid substitutions tolerated. The TRAF6 binding sequence could be more freely substituted, and a TRAF6 consensus sequence is proposed. Stable transfectants expressing selected CD40 mutations were used to define the roles of individual TRAFs in CD40 signaling. Maximal NF-κB and JNK activation through CD40 required both TRAF6 and TRAF1/TRAF2/TRAF3 binding sites. In contrast, p38 MAPK activation resulted primarily from TRAF6 binding to CD40. Competitive TRAF binding and integration of signals from different TRAFs may contribute to a diversity and complexity of CD40 signaling outcomes in different cell types.

MATERIALS AND METHODS

Plasmids and Viruses—The plasmid pGST-CD40 has been described previously (11). Using oligonucleotides 5'-TACATCTGC-TGTCCTCTGAGA-3' and 5'-TTTGGATCCATGGTTCGTCTCCTCTGCACTGAGA-3', the human CD40 gene was amplified by RT-PCR from JY cells and ligated into pGem-T (Promega) to create phCD40/GemT. The Bst-Ze1 fragment from phCD40/GemT was ligated into pcDNA3.1+ digested with NheI and NotI to make phCD40/cDNA. Amino acid substitutions in CD40 were generated using complementary primers with the desired base changes and pGST-CD40 or phCD40/cDNA as templates using the QuickChange site-directed mutagenesis kit (Stratagene). All mutated genes were verified by automated DNA sequencing. The NF-κB reporter plasmid pNF-E-B-Luc was purchased from Stratagene. Recombinant baculoviruses that express TRAF1-CA21, TRAF2-CA21, TRAF3-CA21, and TRAF6-CA21 have been described previously (11).

Protein Expression and Purification—Spodoptera frugiperda (Sf21) cells were maintained and infected as described previously (28) using medium supplemented with 5% heat-inactivated fetal bovine serum (HyClone) and 50 mg/ml gentamicin sulfate (Life Technologies, Inc.). Cells were grown in serum-free medium supplemented with 5% heat-inactivated fetal bovine serum. Cells were maintained and infected as described previously (28) using medium containing 0.4 mg/ml Geneticin (Life Technologies, Inc.). Geneticin-resistant cells were expanded and stained with FITC mouse anti-human CD40 monoclonal antibody (PharMingen). Narrowly gated CD40-expressing cells were sorted into 96-well plates at 1 cell/well with a FACS Vantage (Becton Dickinson). Clones were expanded, and surface CD40 levels were analyzed by flow cytometry on a FACS flow cytometer (Becton Dickinson) as described above. Two independent clonal isolates expressing similar levels of CD40 were selected for each transfected line for subsequent analysis. In all cases, the two independent clones of each cell line responded similarly to CD40 stimulation. Representative results from one isolate are shown for all experiments.

NF-κB Reporter Assay—Three micrograms of the reporter plasmid pNF-E-B-Luc (Stratagene) were transfected into 1 × 10⁶ HEK 293 cells or stable CD40 transfectants of HEK 293 cells in 60-mm dishes using Superfect (Qiagen) according to the manufacturer’s protocol. Forty-eight h after transfection medium was removed and replaced with medium containing 0.4 mg/ml Geneticin (Life Technologies, Inc.). Cells were grown in serum-free medium supplemented with 5% heat-inactivated fetal bovine serum. Cells were maintained and infected as described previously (28) using medium containing 0.4 mg/ml Geneticin (Life Technologies, Inc.). Geneticin-resistant cells were expanded and stained with FITC mouse anti-human CD40 monoclonal antibody (PharMingen). Narrowly gated CD40-expressing cells were sorted into 96-well plates at 1 cell/well with a FACS Vantage (Becton Dickinson). Clones were expanded, and surface CD40 levels were analyzed by flow cytometry on a FACS flow cytometer (Becton Dickinson) as described above. Two independent clonal isolates expressing similar levels of CD40 were selected for each transfected line for subsequent analysis. In all cases, the two independent clones of each cell line responded similarly to CD40 stimulation. Representative results from one isolate are shown for all experiments.

NF-κB and p38 MAPK Activation—One million HEK 293 cells or stable transfectants were plated in 60-mm dishes and incubated overnight at 37 °C. Cells were stimulated for 15 min with medium alone or medium containing 10 μg/ml mouse CD8a-human CD40L fusion protein and 15 μg/ml rat anti-mouse CD8a monoclonal antibody (53-6). After 6 h, cell extracts were prepared and assayed for luciferase activity using the Luciferase assay system (Promega) according to the manufacturer’s protocol.

JNK and p38 MAPK Activation—One million HEK 293 cells or stable transfectants were plated in 60-mm dishes and incubated overnight at 37 °C. Cells were stimulated for 15 min with medium alone or medium containing 10 μg/ml mouse CD8a-human CD40L and 15 μg/ml mouse anti-CD8a. Cells were harvested and lysed in 2% SDS, 50 mM Tris-HCl, pH 6.8, 2% 2-ME. After heating at 56 °C for 5 min, portions of the supernatant were subjected to SDS-PAGE (12% polyacrylamide Tris-glycine; Novex) and transferred to a PVDF membrane (Schleicher & Schuell) by electroblotting. Immunoblot analysis of transferred proteins was performed by incubating membranes with 1 μg/ml anti-p38 (N-20, Santa Cruz), 1 μg/ml anti-JNK (JNK1-FL, Santa Cruz), 1:2000 dilution of anti-Active p38 (Promega), or 1:5000 dilution of anti-Active JNK (Promega), followed by a 1:2000 dilution of Protein A-HRP (Bio-Rad). Visualization of total and activated kinases was performed by chemiluminescence using Pierce SuperSignal chemiluminescent substrate.

RESULTS

Mutational Analysis of TRAF Binding Sites in CD40—Minimal binding sites for TRAF1, TRAF2, TRAF3, and TRAF6 in the human CD40 cytoplasmic domain were previously mapped using peptides from CD40 synthesized on cellulose membranes. Using overlapping peptides and peptides with progressive deletions from the N terminus, C terminus, or both termini, TRAF1, TRAF2, and TRAF3 bound optimally to the sequence 250PVQET, whereas TRAF6 bound to a more membrane-prox...
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Fig. 1. TRAF protein binding to CD40-derived peptides with single amino acid substitutions. Peptides were synthesized in spots on cellulose membranes, each containing a single L- or D-amino acid substitution, as indicated. Membranes were probed with insect cell extracts containing TRAF1-CA21 (A), TRAF2-CA21 (B), TRAF3-CA21 (C), or TRAF6-CA21 (D) as described under “Materials and Methods.” TRAF proteins bound to peptides were visualized by chemiluminescent detection and are shown as scans of the exposed films. The wild type amino acid sequence of each TRAF binding peptide is indicated vertically to the left of the peptide membrane. The amino acid substituted for each position is indicated across the top of each membrane. The first column of peptide spots on the left side of each membrane contains the wild type peptide sequence, as indicated by a dash (“-”). The same peptide membranes were used in A–C for sequential TRAF1, TRAF3, and TRAF2 binding. Membranes were stripped after binding each TRAF with four different buffers as described under “Materials and Methods.”

L-Amino acid or D-amino acid substitutions for residues N-terminal to the 250PVQET sequence minimally affected TRAF1, TRAF2, or TRAF3 binding (Fig. 1, A–C). This indicates these residues were not involved in TRAF interactions with the peptides. The amino acid substituted for each position is indicated across the top of each membrane. The first column of peptide spots on the left side of each membrane contains the wild type peptide sequence, as indicated by a dash (“-”). The same peptide membranes were used in A–C for sequential TRAF1, TRAF3, and TRAF2 binding. Membranes were stripped after binding each TRAF with four different buffers as described under “Materials and Methods.”

D-Amino acid replacements of most residues in the 250PVQET sequence eliminated TRAF1, TRAF2, and TRAF3 binding (Fig. 1, A–C). Peptides with D-Tyr substituted for Pro250 could efficiently bind TRAF1. Peptides with Pro250 replaced by several D-amino acids still maintained TRAF2 binding. TRAF3, but not TRAF1 or TRAF2, bound to peptides with Pro250 replaced by numerous D-amino acids. The 250PVQET sequence could not be substituted with D-amino acids (Fig. 1, A–C), indicating contributions of these side chains to TRAF1, TRAF2, and TRAF3 interactions. Peptides with Thr254 replaced by D-Ser or D-Thr residues maintained TRAF1, TRAF2, and TRAF3 interactions.

L-Amino acid substitutions in the TRAF6 binding peptide indicated there were three amino acid residues critical for TRAF6 binding to the 14-amino acid peptide: Pro233, Glu235, Gln252, and Glu253 could not be replaced by any other L-amino acids, indicating that these residues were critical for TRAF1, TRAF2, and TRAF3 binding to the peptides. Peptides with Thr254 replaced by L-Ser still maintained TRAF1, TRAF2, or TRAF3 binding, but all other substitutions for Thr254 eliminated binding (Fig. 1, A–C). Most L-amino acid or D-amino acid substitutions of Leu255 had little effect on TRAF1, TRAF2, or TRAF3 binding, indicating that this residue was not required for TRAF interactions with peptides from the CD40 cytoplasmic domain. A summary of amino acids allowed at each position and some amino acid replacements that resulted in reduced binding are shown in Table I.
and Phe\textsuperscript{238} (Fig. 1D and Table I). Pro\textsuperscript{232} could only be replaced by L-Ala, and Phe\textsuperscript{238} could be replaced by L-Trp or L-Tyr. Numerous L-amino acid substitutions of the remaining residues of CD40 from positions 230 to 237 could be made without eliminating TRAF6 binding. This suggested that those residues were not making essential contributions to TRAF6 binding to the peptides. Substitution of most residues C-terminal to Phe\textsuperscript{238} with either L-amino acids or D-amino acids minimally affected TRAF6 binding (Fig. 1D), indicating these residues were not required. Most D-amino acid substitutions of the sequence APVQETLGHCQPVTQED eliminated TRAF6 binding to the peptides. These results indicate that the core TRAF6 binding site was not required. Most D-amino acid substitutions of the sequence APVQETLGHCQPVTQED. 

**Table I**

| Consensus binding site |
|------------------------|
| TRAF1  | P/H  | V/I | Q  | E | T/S  |
| TRAF2  | P/H  | V/I | Q  | E | T/S  |
| TRAF3\textsuperscript{a}  | P/G/H | V/I | Q  | E | T/S  |
| TRAF3\textsuperscript{b}  | X   | V/F/I/M | Q/F/W | E | T/S  |
| TRAF6  | Q/N/R | P/A | E | X | X | F/W/Y |

\textsuperscript{a} Boldface font indicates a stronger interaction. The letter \(X\) denotes any amino acid.

\textsuperscript{b} Consensus binding site in the context of the peptide binding site NTAAPFVQETLHGCDQPVQED.

Relative Affinities of TRAFs for Altered CD40 Cytoplasmic Domains—Based on the peptide mutational analyses in Figs. 1 and 2, selected amino acid substitutions designed to differentiate binding of individual TRAFs were engineered in the CD40 cytoplasmic domain. The mutations were tested for interactions with TRAF1, TRAF2, TRAF3, and TRAF6 by expressing and isolating each as a glutathione S-transferase-human CD40 cytoplasmic domain (GST-CD40c) fusion protein and quantitating TRAF binding in a solid-phase binding assay. In this assay, TRAF concentrations were kept constant, and each GST-CD40c fusion protein was titrated over a wide concentration range. Previous results with this assay have shown that receptors that bind TRAFs weakly, such as OX40, only bind TRAF proteins at relatively high GST-cytoplasmic domain concentrations. In contrast, receptors that bind TRAFs strongly, such as CD40, give strong signals at 10–20-fold lower concentrations of GST fusion protein.\textsuperscript{3}

To eliminate TRAF binding to the 250PVQET (TRAF1/ TRAF2/ TRAF3 binding) sequence, the T254A mutation was produced. Confirming and extending previous reports (14, 15), GST-CD40c T254A had no significant binding of TRAF1, TRAF2, or TRAF3 (Fig. 3). This was confirmed in GST-CD40c coprecipitation assays using glutathione-Sepharose beads (data not shown). GST-CD40c T254A bound TRAF6 similar to wild type GST-CD40c consistent with an earlier study (15). To target the 231QEPQEINF (TRAF6 binding) sequence, two mutations were produced, one designed to eliminate and one designed to enhance TRAF6 binding. From the mutational analyses, a GST-CD40c fusion protein with the paired P233G/E235A substitutions was predicted to eliminate TRAF6 binding. As anticipated, GST-CD40c P233G/E235A did not interact with TRAF6 (Fig. 3D). Interestingly, TRAF1, TRAF2, and TRAF3 exhibited increased binding to GST-CD40c containing the P233G/E235A substitution. TRAF6 binding to GST-CD40c containing the second mutation in the 231QEPQEINF sequence, N237D, was increased over 25-fold relative to wild type GST-CD40c (Fig. 3D). TRAF1, TRAF2, and TRAF3 binding to the GST-CD40c N237D fusion protein was also significantly increased (3–4-fold). GST-CD40c fusion proteins that contained combinations of mutations in the 250PVQET and the 231QEPQEINF TRAF binding sites were also produced. The triple substitution P233G/E235A/T254A GST-CD40c exhibited no significant binding to TRAF1, TRAF2, TRAF3, or TRAF6 (Fig. 3). This assay was also used to verify that GST-CD40c fusion proteins containing the T254A, P233G/E235A, N237D, and P233G/E235A/T254A mutations exhibited no binding to TRAF5 (data not shown). GST-CD40c containing combined N237D/T254A mutations had no significant binding of TRAF1, TRAF2, and TRAF3, but still bound TRAF6 approximately 25-fold better than wild type GST-CD40c (Fig. 3). The GST-CD40c-TRAF binding results demonstrated that although the sequences 231QEPQEINF and 250PVQET in CD40 appear to be independent binding sites for unique subsets of TRAFs based on deletional (15, 27) and peptide scanning (11) analyses, point mutations in the 231QEPQEINF binding site altered TRAF binding to the 250PVQET binding site.

Based on the mutational analyses in Figs. 1 and 2, several other mutations in the CD40 cytoplasmic domain were made and expressed as GST-CD40c fusion proteins. These were purified and tested for TRAF binding in the solid-phase binding assay. GST-CD40c containing a P250H mutation had slightly increased TRAF3 and TRAF6 binding, but had almost background levels of TRAF1 and TRAF2 binding (data not shown). Binding of GST-CD40c containing a Q252E mutation to TRAF6

\textsuperscript{3} T. T. A. Dang, S. S. Pullen, J. J. Crute, and M. R. Kehry, manuscript in preparation.
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**Fig. 2.** TRAF2 and TRAF3 binding to CD40-derived peptides with single L-amino acid substitutions. Peptides synthesized with single L-amino acid substitutions were probed with insect cell extracts containing TRAF2-CA21 (A) or TRAF3-CA21 (B) as described under “Materials and Methods” and the legend of Fig. 1. The wild type amino acid sequence of the TRAF binding peptide is indicated vertically to the left of the peptide membrane. The amino acid substituted for each position is indicated across the top of each membrane. The first column of peptide spots on the left side of each membrane contains the wild type peptide sequence, as indicated by a dash (−). The same peptide membrane was used in A and B for sequential binding of TRAF2 and TRAF3. The membrane was stripped after TRAF2 binding with the two 100 mM 2-ME-containing buffers as described under “Materials and Methods.”

**Fig. 3.** Solid-phase binding of TRAFs to wild type and mutated GST-CD40c proteins. Serial dilutions of GST-CD40c fusion proteins were bound to glutathione-coated 96-well plates as described under “Materials and Methods.” Insect cell extracts diluted in binding buffer and containing approximately 1 μg/ml each TRAF1-CA21 (A), TRAF2-CA21 (B), TRAF3-CA21 (C), or TRAF6-CA21 (D) were incubated in the wells and detected with biotin-CA21 antibody as described under “Materials and Methods.” No signal was generated in the assay with biotin-CA21 when wells were coated with GST-CD40c or not coated (data not shown). The molecular mass of each mutated GST-CD40c fusion protein was verified by electrospray ionization mass spectrometry. Each point is the mean of duplicate wells. Figure is representative of three independent experiments.

was identical to that of wild type GST-CD40c. However, this mutation decreased binding of GST-CD40c to TRAF3 approximately 3-fold and completely eliminated TRAF1 and TRAF2 binding (data not shown). These results with GST-CD40c containing either P250H or Q252E alterations were consistent with the mutational analysis in Fig. 2 and support the idea that amino acids C-terminal to the 250PVQET sequence contributed to CD40-TRAF3 interaction but not to CD40-TRAF1 or CD40-TRAF2 interactions. Based on the data in Fig. 1D, an I236W substitution was also produced in GST-CD40c. As expected, GST-CD40c I236W bound to TRAF6 approximately 4-fold better than to wild type GST-CD40c; however, its binding to TRAF1, TRAF2, and TRAF3 was similar to that of wild type GST-CD40c. The results with these mutations are consistent with and support results obtained in the peptide-based mutational analyses.

**Signaling Functions of CD40 Mutations—**To study signaling outcomes from altered TRAF binding to the CD40 mutations, each mutation characterized in Fig. 3 was introduced into full-length human CD40. HEK 293 cells were demonstrated by RT-PCR (TRAF1, TRAF3, TRAF4, and TRAF5) and immunoblot analyses (TRAF2 and TRAF6) to endogenously express all six known TRAF molecules (data not shown). Stable transfectants of HEK 293 cells were generated that expressed the wild type human CD40 receptor or each of the five mutations characterized in Fig. 3. Cells were cloned by flow cytometry, and clones that expressed similar levels of CD40 on the cell surface were chosen for further studies (Fig. 4). To confirm in vitro binding results with GST-CD40c, TRAF associations with CD40 in cells were examined by coimmunoprecipitation. Coimmunoprecipitations of TRAF2, TRAF3, and TRAF6 with wild type CD40 or the CD40 cytoplasmic domain mutants stably expressed in HEK cell lines were completely consistent with the specificity obtained using in vitro CD40-TRAF binding assays (Fig. 3) (data not shown).

To measure CD40-dependent NF-κB activation, each clone was transfected with an NF-κB-luciferase reporter plasmid and 24 h later stimulated with soluble CD80-human CD40L and anti-CD80. Luciferase activity was assessed 6 h after CD40L stimulation. HEK 293 cells and a stable cell line transfected with the empty expression vector were used as negative controls and had similar basal levels of NF-κB activity. There was no increase in NF-κB activity upon stimulation of HEK 293 or vector-transfected cells with CD80-CD40L fusion protein (Fig. 5). Clones expressing the wild type CD40 receptor displayed increased basal levels of NF-κB activity when compared with HEK 293 cells and vector-transfected clones. After stimulation with CD80-CD40L, the wild type CD40 transfectants showed a 4-fold increase in NF-κB activity over their basal level (Fig. 5). In contrast, clones expressing the CD40 T254A mutation had basal levels of NF-κB activity similar to HEK 293 and vector-transfected clones. CD80-CD40L stimulation of the CD40 T254A clones resulted in a 150-fold enhancement of NF-κB activity over their basal level (Fig. 5). Clones expressing the wild type CD40 receptor displayed increased basal levels of NF-κB activity when compared with HEK 293 cells and vector-transfected clones. After stimulation with CD80-CD40L, the wild type CD40 transfectants showed a 4-fold increase in NF-κB activity over their basal level (Fig. 5). In contrast, clones expressing the CD40 T254A mutation had basal levels of NF-κB activity similar to HEK 293 and vector-transfected clones. CD80-CD40L stimulation of the CD40 T254A clones resulted in a 150-fold enhancement of NF-κB activity over their basal level (Fig. 5).
levels of NF-κB mutation with no detectable TRAF6 binding had basal activation over basal levels. Cells expressing the CD40 P233G/TRAF6 binding showed basal levels of NF-κB activity, although the absolute signal was 2–3-fold lower than in the wild type CD40 transfectants. Cells expressing the CD40 N237D receptor with increased TRAF6 binding exhibited the highest basal and CD40L-stimulated levels of NF-κB activation (Fig. 5). The increase in NF-κB activation after CD40L stimulation was 2.5-fold. Cells expressing the CD40 N237D/T254A receptor showed increased basal NF-κB activity when compared with cells expressing the CD40 T254A mutation. The basal NF-κB activity was 2–3-fold lower than for the wild type CD40 transfectants. Stimulation of the CD40 N237D/T254A clones with CD40L resulted in a 5-fold increase in NF-κB activity over basal levels. Cells expressing the CD40 P233G/E235A mutation with no detectable TRAF6 binding had basal levels of NF-κB activity approximately 2-fold lower than cells expressing wild type CD40. After CD40L treatment, cells expressing the CD40 P233G/E235A receptor had levels of NF-κB activation 3-fold lower than CD40L-treated wild type CD40 transfectants. Cells expressing the CD40 P233G/E235A/T254A mutation that gave no detectable TRAF1, TRAF2, TRAF5, or TRAF6 binding showed basal levels of NF-κB activity similar to HEK 293 and vector transfectants. Surprisingly, after CD40L treatment, there was an 8-fold increase in NF-κB activity (Fig. 5). The identity of the CD40 P233G/E235A/T254A sequence in the transfectant was confirmed by RT-PCR and DNA sequence analysis. This indicates that although TRAF1, TRAF2, TRAF5, TRAF6, and TRAF6 did not bind to the CD40 P233G/E235A/T254A receptor, CD40L-dependent activation of NF-κB was reduced but not eliminated. Together, the NF-κB signaling results demonstrate that both TRAF binding sites in CD40 contribute to NF-κB activation.

Previous studies demonstrated that JNK activation also results from stimulation of CD40 signaling (4–7). The ability of each mutated CD40 receptor to mediate JNK activation was determined by stimulating each of the stable transfectants with CD8α-CD40L fusion protein, and anti-CD8α. After a 15-min stimulation, cell lysates were prepared and subjected to immunoblot analysis using an antibody that recognizes total JNK1 and JNK2 isoforms or an antibody that recognizes activated, dual phosphorylated JNK1 and JNK2 isoforms. HEK 293 cells had very low levels of constitutively activated JNK, and there was no increase in activated JNK levels after stimulation with CD40L (Fig. 6A). The transfectant expressing wild type CD40 had similar basal levels of activated JNK as untransfected HEK 293 cells, and treatment with CD40L significantly increased the levels of activated JNK1 and JNK2 (Fig. 6A). Stimulation of the CD40 T254A-expressing cells with CD40L resulted in significantly reduced JNK activation when compared with cells with wild type CD40. Some residual JNK activation was still observed in the CD40 T254A cells. Cells expressing the CD40 N237D receptor with increased TRAF6 binding had elevated levels of JNK activation in the absence of CD40L stimulation. After CD40L treatment, cells expressing CD40 N237D showed the highest levels of activated JNK when compared with all of the other cell lines (Fig. 6A). CD40L stimulation of cells expressing the CD40 N237D/T254A receptor resulted in levels of JNK activation similar to cells with wild type CD40. Cells expressing the CD40 P233G/E235A receptor showed dramatically reduced JNK activation after CD40L treatment. Consistent with a complete absence of TRAF1, TRAF2, TRAF3, and TRAF6 binding by CD40 P233G/E235A/T254A, no JNK activation was observed after CD40L treatment of CD40 P233G/E235A/T254A-expressing cells (Fig. 6A). Similar levels of JNK1 and JNK2 isoforms were present in lysates from all the cell lines (Fig. 6B). Together, these results demonstrate that both TRAF binding sites in CD40 are involved in mediating JNK activation. However, the TRAF6 binding site appeared to be more critical for JNK activation.

Another outcome of CD40 signaling in B lymphocytes is activation of p38 MAPK (8, 9). Therefore, activation of p38 MAPK in the CD40 HEK 293 transfectants was also examined. After 15 min of stimulation with CD8α-CD40L, cell lysates were prepared and subjected to immunoblot analysis using an antibody that recognizes total p38 MAPK or an antibody that recognizes the activated, dual phosphorylated form of p38.
amino acid substitutions of the 250PVQET residues were tolerated with 10 μg/ml CD8a-human CD40L and 15 μg/ml rat anti-mouse CD8α (+) for 15 min were separated by SDS-PAGE on a 12% polyacrylamide gel and electroblotted to a PVDF membrane. Detection of phosphorylated active JNK1 and JNK2 (A) and total JNK1 and JNK2 isoforms (B) was performed by immunoblotting as described under “Materials and Methods.” Migration of JNK1 and JNK2 isoforms are indicated by arrows. A slight decrease in mobility of phosphorylated forms of JNK1 could be seen with the antibody that detected total JNK1 and JNK2 (B), confirming the results obtained in A with the antibody that recognizes only phosphorylated JNK1 and JNK2. Figure is representative of two independent experiments on one representative clone of each transfectant.

MAPK. Similar low levels of active p38 MAPK were detected in unstimulated or CD40L stimulated HEK 293 cells (Fig. 7A). Transfectants with wild type CD40 showed a significant increase in active p38 MAPK following CD40L treatment. After CD40L stimulation, cells expressing CD40 T254A receptor showed levels of p38 MAPK activation similar to wild type CD40 transfectants (Fig. 7A). Cells expressing CD40 N237D and CD40 N237D/T254A receptors also potently activated p38 MAPK after stimulation with CD40L. In contrast, after CD40L treatment, cells containing the CD40 P233G/E235A receptor showed a significant reduction in the amount of p38 MAPK activated. No CD40L-dependent increase in p38 MAPK activation occurred in lyses from the CD40 P233G/E235A/T254A receptor. Similar levels of total p38 MAPK were present in lyses from all the cell lines (Fig. 7B). These results suggest that the TRAF6 binding site is critical for p38 MAPK activation through CD40. These findings and the TRAF6 binding data in Fig. 3 predict that p38 activation mediated by the CD40 N237D mutations would be higher than that of wild type CD40. A reason this result was not observed in Fig. 7 may be that maximal p38 activation was already achieved by signaling through wild type CD40, and it was therefore not possible to observe increases in p38 activation. Results of TRAF binding and signaling through NF-κB, JNK, and p38 MAPK pathways for wild type CD40 and the five CD40 point mutants are summarized in Table II.

DISCUSSION

Previously, the sequence 250PVQET in the human CD40 cytoplasmic domain has been demonstrated to be necessary and sufficient for interactions with TRAF1, TRAF2, and TRAF3 (11). Additionally, the sequence 231QEPQEFINF appeared sufficient for TRAF6 binding (11). Our peptide-based mutational analysis around the 250PVQET sequence demonstrated that TRAF1, TRAF2, and TRAF3 have similar binding specificities for this core sequence (Fig. 1, A–C). This is interesting in view of the different affinities of TRAF1, TRAF2, and TRAF3 for the CD40 cytoplasmic domain (11). Since few t-amino acid substitutions of the 250PVQET residues were tolerated, the side chain orientation of the residues is critical for TRAF1, TRAF2, and TRAF3 binding. From these studies a consensus core binding site for TRAF1, TRAF2, and TRAF3 in the context of CD40 can be defined as (P/H)(V/I)QE(T/S) (Table I). This extends the previously proposed binding sequence, PQQX(T/S) (26). Because in our mutational analyses only single amino acid replacements were tested, other multiple combinations of amino acid substitutions may also support TRAF1/ TRAF2/TRAF3 binding. This is likely since binding sites for TRAF1, TRAF2, and/or TRAF3 within other receptors, for example TNFR2, ATAR, and a second site in CD30, do not exactly fit the proposed consensus sequence.

Flanking sequences also modulated and generated some selectivity of TRAF1/TRAF2/TRAF3 interactions. Sequences C-terminal to the 250PVQET sequence and previously implicated in facilitating TRAF binding (11) were able to alter the binding specificity of TRAF3 but not TRAF1 or TRAF2 by increasing flexibility in the TRAF3 recognition sequence, 250PVQET. This suggests that although sequences C-terminal to the 250PVQET are not sufficient to support TRAF3 binding, they may alter the manner in which TRAF3 interacts with the 250PVQET sequence. Additionally, the substitution P250H, which had little effect on TRAF1 and TRAF2 binding in the context of peptides, eliminated binding of each to GST-CD40c. In contrast, TRAF3 binding to GST-CD40c P250H was relatively unaffected. Thus, each TRAF binding site in a receptor appears to be defined by a core binding sequence in the context of flanking sequences.

Using the peptide-based amino acid substitutions to form a consensus TRAF6 binding sequence, we predict the TRAF6 binding site in mouse CD40 to be 234KQDPQEEMEDY (Fig. 8). A region of mouse CD40 containing this sequence has been shown to be required for interaction with mouse TRAF6 (15). In this region, the human and mouse CD40 sequences are only partially conserved, with 44% identical residues and a single amino acid insertion in the mouse sequence. This lack of homology is consistent with the ability to optimize this region for TRAF6 binding by two amino acid replacements in human CD40 (N237D and I236W) that significantly increased TRAF6 binding. TRAF6 also interacts with interleukin-1 receptor-associated kinase (IRAK) and receptor activator of NF-κB (RANK). Using the mutational analysis on the 230KQEPQEFINFPPDDL peptide (Fig. 1D) and a comparison of the mouse and human CD40 TRAF6 binding sequence (Fig. 8), we predict the TRAF6 recognition sequences in human and mouse RANK and human IRAK (Fig. 8). For RANK, the predicted TRAF6 binding

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CD40-TRAF Interactions

**TABLE II**

| CD40 receptor | TRAF1<sup>a</sup> | TRAF2 | TRAF3 | TRAF6 | NF-κB<sup>b</sup> | JNK | p38 |
|---------------|------------------|-------|-------|-------|------------------|-----|-----|
| WT            | +                | +     | +     | +     | +                | +  | +   |
| T254A         | +                | +     | +     | +     | +                | +  | +   |
| N237D         | +                | +     | +     | +     | +                | +  | +   |
| N237D/T254A   | -                | -     | +     | +     | +                | +  | +   |
| P233G/E235A   | +                | +     | +     | +     | +                | +  | +   |
| P233G/E235A/T254A | +                | +     | +     | +     | +                | +  | +   |

<sup>a</sup> Relative binding (indicated by a +) or a lack of binding (indicated by a −) of TRAFs to CD40 receptors.

<sup>b</sup> Degree of activation of signaling pathways (indicated by a +) or the lack of activation (indicated by a −) for cell lines expressing each receptor.

Homo sapiens (hCD40)<br>
Mouse (mCD40)<br>
Human (hRANK)<br>
Mouse (mRANK)<br>
Human (hTRAF6)

**Fig. 8. Predicted TRAF6 binding sites.** The binding site on human CD40 for TRAF6 is aligned by homology with sequences of proteins known to interact with TRAF6. Conserved residues from the mutational analysis on CD40 are highlighted in black. Numbers indicate the amino acid residues of each protein.

Site is located within a region demonstrated previously to be involved in TRAF6 binding (39) and NF-κB and JNK activation (39, 40). The predicted TRAF6 binding site in RANK is 78% identical between mouse and human proteins (Fig. 8). A predicted TRAF6 binding site in IRAK is positioned C-terminal to the kinase domain (24) (Fig. 8). Interestingly, RANK, IRAK, and mouse CD40 have a C-terminal Tyr residue instead of a Phe in the predicted TRAF6 binding site. It is possible that tyrosine phosphorylation could modulate TRAF6 binding to these other proteins.

Selected amino acid substitutions were made in the context of the full-length CD40 cytoplasmic domain and characterized in *vitro* and in cells to confirm that the mutations individually eliminated TRAF interactions with either or both of the two TRAF binding sites (Table II). Two substitutions in the TRAF6 binding site, N237D and P233G/E235A, increased TRAF1, TRAF2, and TRAF3 binding to CD40 (Fig. 3). This suggests that although TRAF1, TRAF2, and TRAF3 do not independently bind to the TRAF6 site in CD40 (11), they may make contacts in the TRAF6 binding region. Thus, it is possible that the binding of a TRAF to either of the binding sites in CD40 could sterically prevent simultaneous TRAF interaction with the other site. The binding and competition of TRAF proteins to a more native multimerized form of CD40 engaged by CD40L remains to be tested. A competitive interaction of TRAFs with cross-linked CD40 suggests that the levels of individual TRAFs in different cell types could mediate specificity in outcomes of CD40 signaling. Alternatively, the TRAF6 binding region may be folded so as to be in close proximity to the TRAF1/TRAF2/TRAF3 binding region and form a tertiary structure that optimally interacts with TRAF1, TRAF2, and TRAF3.

Signaling by specific CD40 mutants was compared with wild type CD40 in stably transfected HEK 293 clones (results summarized in Table II). Since HEK 293 cells expressed all six identified TRAFs and little if any CD40, it was possible to study CD40 signaling using endogenous levels of signaling proteins. The high basal level of NF-κB activation in clones expressing wild type CD40 was reduced to that of untransfected cells by expressing the T254A substitution that eliminated TRAF1/TRAF2/TRAF3 binding. Due to high levels of CD40 expression in the transfecants, TRAF2 and TRAF3, which bind to CD40 better than TRAF1 and TRAF6<sup>4</sup> (11), may interact with CD40 and mediate NF-κB activation in the absence of receptor cross-linking. Since TRAF2 appears dispensable for NF-κB activation (17, 18), basal NF-κB activation could be mediated by TRAF5 that can be recruited to CD40 as a hetero-oligomer with TRAF3 (11). The cross-linking independent NF-κB activation also is consistent with increased basal levels of NF-κB activity in cells expressing the CD40 N237D receptor with increased affinity for TRAF6 and TRAF2.

The dramatic increase in NF-κB activity upon CD40L stimulation of the CD40 T254A clones is consistent with previous findings (41), indicating that TRAF6 is a potent activator of NF-κB through CD40. Reduced levels of NF-κB activation were found in clones expressing CD40 with TRAF6 binding eliminated (P233G/E235A). This further confirms an important role for TRAF6 in mediating NF-κB activation through CD40. Overall, the results suggested that NF-κB activation through CD40 is mediated by TRAF6 as well as possibly TRAF2 and/or TRAF5. The significant CD40-dependent NF-κB activation (yet the complete absence of JNK and p38 MAPK activation) in clones expressing the P233G/E235A/T254A mutation was unexpected. This suggests that there may be another mediator of NF-κB activation through CD40 in addition to the known TRAF proteins.

JNK activation through CD40 (4–9) could be mediated by TRAF2, TRAF5, and/or TRAF6 (17, 18, 21, 22, 42, 43). Both TRAF binding sites in CD40 were required for maximal JNK activation (Fig. 6 and Table II). Mutations that individually eliminated TRAF binding to either site in CD40 only partially reduced JNK activation after CD40 stimulation. Mutations that eliminated TRAF binding to both sites eliminated JNK activation. This suggests that all JNK activation through CD40 is mediated by CD40-TRAF interactions. In a previous study, TRAF2 activated p38 MAPK through TNFR1 in HEK 293 cells (19). In our analysis, a mutation that eliminated TRAF1, TRAF2, and TRAF3 binding (T254A) had little if any effect on p38 MAPK activation (Fig. 7). Instead, primarily TRAF6 binding appeared to be required for p38 MAPK activation through CD40. It is possible that differences in TRAF expression could account for these different findings since TRAF2 was overexpressed in the earlier study (19). If TRAF6 is of critical importance in mediating p38 MAPK-dependent events resulting from CD40 signaling, it would be expected that TRAF6 is essential for CD40-mediated events in B cells that are dependent upon p38 MAPK activation such as proliferation and ICAM-1 and CD40 induction (8).

Our studies are consistent with previous studies that used deletions and selected substitutions to map regions within the CD40 cytoplasmic domain required for signaling. Additionally, some confusing findings in previous work are explained. A role for the 260PVTQED sequence in contributing to TRAF3 binding to the 250PVQET sequence may explain why deletion of the C-terminal 22 residues of CD40, which includes the sequence 260PVTQED but not 250PVQET, caused a 50% reduction in NF-κB activation (41), reduced LFA-1 and ICAM-1 induction, and eliminated CD23, B7-1, and Fas induction (44). This may reflect a role for TRAF3/TRAF5 hetero-oligomers in these events. The difference in the effects of this deletion on different
responses may indicate that a critical balance of TRAF1/ TRAF2 versus TRAF3/TRAF5 interactions may be required to mediate each outcome. Previously, removal of the C-terminal 32 residues of CD40, which includes the 250PVQET sequence, eliminated all signaling through CD40 (41, 44). However, in that study, human CD40 deletions were tested in a mouse cell line. It is possible that, since the TRAF6 binding sites are not eliminated all signaling through CD40 (41, 44). However, in contrast, in another study, CD40 with the C-terminal 31 residues deleted still mediated NF-κB activation (45), consistent with TRAF6 binding to the 233QEPQEINF sequence. Previous signaling studies on the CD40 T254A receptor (41) are confirmed by our finding of a reduction in NF-κB activation. Additionally, the T254A substitution was found to reduce antibody secretion in B cells, reduce LFA-1 and ICAM-1 induction, and eliminate CD23, B7-1, and Fas induction (44, 46). The downstream outcomes of CD40 signaling that were partially inhibited by the T254A replacement are most likely dependent upon events such as NF-κB, JNK, and p38 MAPK activation that are also mediated by TRAF6. Because NF-κB and JNK activation are also mediated through the 250PVQET sequence, a certain threshold of signaling or combination of signals that can only be achieved by signaling through both TRAF binding sites may be required for some CD40-mediated effects.

This work demonstrates that outcomes of signaling through CD40 result from a combination of the contribution of individual TRAFs interacting with the CD40 cytoplasmic domain. It is not yet known whether the extent of CD40 cross-linking could facilitate binding of some TRAFs but not others. Diversity in the outcome of CD40 signaling in different cell types could be achieved through differential regulation of levels of different TRAFs as well as by regulating linkages to downstream signaling pathways. Further studies will be necessary to determine the distinct physiological outcomes of CD40 signaling using different cell types that normally express CD40.

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