CD40 Signaling through a Newly Identified Tumor Necrosis Factor Receptor-associated Factor 2 (TRAF2) Binding Site*

Li-Fan Lu, W. James Cook‡, Ling-Li Lin, and Randolph J. Noelle§

From the Department of Microbiology and Immunology, Dartmouth Medical School, Lebanon, New Hampshire 03756

Tumor necrosis factor receptor-associated factors (TRAFs) belong to a family of adapter proteins that are involved in tumor necrosis factor receptor superfamily signaling. It has been shown that the recruitment of TRAFs to the CD40 cytoplasmic tail is essential for CD40-mediated B cell responses. However, it has also been shown that some early B cell responses, such as up-regulation of cell surface molecules and B cell proliferation are only marginally impaired by the disruption of previously defined TRAF binding sites (Ahonen, C., Manning, E., Erickson, L. D., O’Connor, B. P., Lind, E. F., Pullen, S. S., Kehry, M. R., and Noelle, R. J. (2002) Nat. Immunol. 3, 451–456; and Manning, E., Pullen, S. S., Souza, D. J., Kehry, M., and Noelle, R. J. (2002) Eur. J. Immunol. 32, 39–49). In this report, we identify a second TRAF2 binding site in the CD40 C terminus. The binding motif "SVQE" fits into the major TRAF2 binding consensus sequence, and its disruption resulted in the loss of remaining CD40 functions. Hence, like CD30, the CD40 cytoplasmic tail contains two distinct and functionally important TRAF2 binding sites.

CD40, a member of the tumor necrosis factor receptor superfamily, was first identified and functionally characterized on B lymphocytes. Engagement of CD40 by its ligand, CD154, on activated helper T lymphocytes initiates signals that trigger up-regulation of a number of B cell surface molecules including regulatory molecules such as CD23 and co-stimulatory molecules such as B7.1, B7.2 (1, 2). Engagement of CD40 also elicits clonal B cell expansion, germinal center formation, generation of memory B cells, isotype switching, affinity maturation, and other events necessary for efficient thymus-dependent humoral immunity (3). CD40, like other tumor necrosis factor receptor superfamily members, recruits several different tumor necrosis factor receptor-associated factors (TRAFs) to its cytoplasmic tail after multimerization by its ligand (4–6). CD40 transduces signals via these adapter proteins to mediate the activation of multiple signaling pathways including NFκB, c-Jun N-terminal kinase, and p38 mitogen-activated protein kinase, which in turn phosphorylate and activate downstream transcription factors (7–10).

To define the role that TRAFs play in CD40-mediated B cell activation, a number of groups have engineered mutations into the cytoplasmic domain of CD40 to selectively disrupt the binding of specific TRAFs. In both B cell lines and transgenic mice in which the canonical TRAF2, TRAF3, and TRAF6 sites have both been disrupted (ΔT2,3,6), some CD40-mediated signaling and function were still intact. Other reports have documented that even with the mutations in the canonical TRAF binding sites, the binding of TRAFs to the CD40 cytoplasmic tail could still be detected (11, 12). Taken together, these data suggest that TRAF binding to a site mapping outside of the established sites could be responsible for residual signaling in which the canonical TRAF binding sites have been disrupted.

In this report, we have identified a new functional domain within the highly conserved C terminus of CD40 cytoplasmic tail. We have shown that this domain contains a functional TRAF2 binding site. Site-directed mutagenesis of the second TRAF2 binding site has shown that ablation of TRAF2 binding results in loss of biological activity in this domain. The importance of this site in B cell activation and its function relative to the dominant TRAF2 site is discussed.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—The murine B cell line M12.4.1 was maintained in RPMI 1640 medium with 10% fetal bovine serum, 10 μM β-mercaptoethanol, and antibiotics. Stable transfections of B cells were carried out using electroporation as described previously (2). G418-resistant clones were isolated by magnetic cell sorting (Miltenyl Biotech) with biotin-conjugated anti-human CD40 antibody and were further analyzed for surface expression of chimeric CD40 by using a fluorescence-activated cell sorter. Polyclonal transfectants with chimeric CD40 expression were used for experiments to prevent clonal variation.

Construction of Chimeric CD40 with a Different Cytoplasmic Tail—All of the chimeric CD40 constructs were constructed using PCR. Following sequence verification, fragments were subcloned using EcoRI sites into vector pDOI-5 for expression under the major histocompatibility complex class II promoter as described previously (1, 2). In constructs Del80 and Del260SEAA, a SalI site was used to make the terminal domain.

Antibodies and Reagents—Polyclonal rabbit antibody to mouse TRAF2 (C-20) and horseradish peroxidase-conjugated donkey anti-goat IgG secondary antibody were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). EZ-Link sulfo-NHS-LC-biotin and goat anti-biotin antibody were purchased from Pierce. Monoclonal antibody S2C6 to human CD40 was kind gift of S. Paulie (Stockholm University, Stockholm, Sweden). Phycoerythrin-conjugated antibody to CD23 was purchased from BD Pharmingen (San Diego, CA). Horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody was from Vector Labs (Burlingame, CA). shCD154 fusion protein has been described in Ref. 1. Supernatants from mCD154/mCD8 transfected S99 cells was a kind gift of K. Marilyn (Roche Applied Science).

Up-regulation of CD23—M12 cells (5 × 10^5) transfected with differ-
ent chimeric CD40 constructs were stimulated in 250 µl of culture medium for 24 h (37 °C) with medium, shCD154 (200 ng/ml), and smCD154 (1.50 dilution of supernatants from mCD154/mCD8 transfected SF9 cells). Cells were then washed and stained with antibodies for analysis by flow cytometry.

Immunoprecipitations and Western Blotting—M12 cells (2.5 × 10⁶) transfected with different chimeric CD40 constructs were biotinylated following the manufacturer’s instructions (Pierce) and then stimulated in 1 ml of culture medium for 15 min (37 °C) with shCD154 (200 ng/ml). Cells were washed once with cold phosphate-buffered saline and then resuspended in 250 µl of lysis buffer (10 mM sodium phosphate, pH 7.2, 150 mM NaCl, 2 mM EDTA, 1% IGEPAL, 1 mM dithiothreitol) in the presence of protease inhibitors and phosphatase inhibitors. After incubation 30 min (4 °C), cleared supernatants were precleared for 30 min at 4 °C on a rotator with protein G-agarose beads (Pierce) and then incubated for 1 h at 4 °C with protein G-agarose beads preconjugated with anti-hCD40 monoclonal antibody. Beads were washed four times with cold phosphate-buffered saline with protease inhibitors and phosphatase inhibitors and then once with cold Tris buffer (pH 7.2). Immunoprecipitated proteins were separated by SDS-PAGE and then transferred onto nitrocellulose membranes. A chemiluminescent substrate (Pierce) was used to detect horseradish peroxidase-labeled secondary antibodies on Western blots.

**Yeast Two-hybrid Screening.—**A murine splenic B cell library was made by using BD Matchmaker Library Construction and Screening kits (Clontech Laboratories). Library screening by the yeast two-hybrid method was performed according to the manufacturer’s instructions. Briefly, positive transformants were identified by growth on SD-His, -Leu, and -Trp plates and blue color in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Clontech Laboratories). Putative clones were grouped by restriction mapping of PCR products generated for insert DNAs. Representatives of each group were reconfirmed by retransformation into yeast containing the original bait plasmid or the negative control empty vector and human lamin C as instructed by Clontech. Putative interacting clones that tested positive at this step were sequenced and blasted against GenBank™ sequences. Protein-protein interactions were further analyzed by β-galactosidase assay (PB Biosystems).

**RESULTS**

**The Highly Conserved C Terminus of the CD40 Cytoplasmic Tail Can Induce the Up-regulation of CD23- and CD40-induced Growth Arrest—**Previous studies have shown that in B cells from Tg mice, or in B cell lines engineered to express mutations in the TRAF2, -3, and -6 sites (ΔT2,3,6), many of the early CD40-mediated B cell responses are still intact (1, 2). These data suggested that early B cell activation via CD40 was independent of the TRAF binding sites. The identity of the CD40 functional domain that was responsible for the activity of the ΔT2,3,6 cytoplasmic tail was deduced by site-directed mutagenesis and/or deletional analysis. The murine B cell line (M12.4.1) was engineered to express a chimeric human/mouse CD40 protein with different deletions and site-directed mutations of cytoplasmic tail (Fig. 1A). As described previously, the use of a chimeric molecule with a murine cytoplasmic domain ensures the intact species-specific cytoplasmic protein interactions, whereas the use of a human extracellular domain allows the selective engagement of the transfected protein in the presence of the endogenous murine CD40 protein (2). For all analyses, M12 cell lines expressing the human/mouse chimeric constructs were stimulated with human CD154 (for triggering the human/mouse chimeric expressed receptor) or mCD154 (as a control to trigger via the endogenous murine receptor).

A series of mutant chimeric CD40 molecules were transfected into M12 cells to determine their capacity to transduce signals. To evaluate the functional capacity of each of these mutant chimeric proteins in signal transduction, CD40-induced up-regulation of CD23 was used as a measurement. CD40-induced up-regulation of CD23 has been used repeatedly to evaluate the integrity of CD40 signaling (1, 2, 13). Moreover, signaling through CD40 causes proliferation of normal B cells but causes growth arrest of B lymphomas (14). As previously
reported, M12 cells where the TRAF2, -3, and -6 sites were mutated (ΔT2,3,6) still up-regulated CD23 and induced growth arrest in response to CD40 engagement, indicating that the site(s) outside the canonical TRAF binding sites was/were responsible for these early activities. Therefore, the ability of M12 cell lines to express different CD40 mutants to up-regulate CD23 and to inhibit cell growth was functionally accessed. Extensive deletion of the cytoplasmic domain (STOP222) was used as a negative control. After evaluating the functional activities of a series of CD40 constructs (data not shown), grafting of the highly conserved C terminus of CD40 (residues 261–289; Del260) onto the STOP222 “backbone” appeared to restore activity (Fig. 2). As an internal control, M12 cells expressing different CD40 constructs showed comparable activities signaling through endogenous murine CD40 protein (Fig. 2). Inspection of the C-terminal domain revealed a putative TRAF2 binding site, SVQE (residues 273–276). To determine whether this putative TRAF2 binding site was responsible for the up-regulation of CD23 and growth arrest, site-directed mutagenesis was performed. A CD40 molecule with this site mutated (Del260 SEAA) was transfected into M12 cells and its activity tested. Mutagenesis of this site completely ablated both up-regulation of CD23 and growth arrest induced by shCD154 (Fig. 2, A and B).

CD154-induced Binding of TRAF2 to the C Terminus of CD40—TRAF2 binding to the C terminus of CD40 was directly assessed by immunoprecipitation of the CD40 receptor complex. After treatment with shCD154, lysates of M12 cells were subjected to immunoprecipitation with anti-CD40 monoclonal antibody and analyzed by a fluorescence-activated cell sorter. B viability assessed by propidium-iodide staining in addition to forward scatter and side scatter profiles. Data are shown as the percent reduction in the live cell population as compared with an untreated control. Results are representative of at least three independent experiments. WT, wild type.

engineered CD40 cytoplasmic tails that lacked all known TRAF binding sites and expressed these as baits in yeast two-hybrid screening (Fig. 1B). Using a fragment of CD40 consisting of the highly conserved C terminus (Del260) from a total $-5 \times 10^5$, $-240$ positive clones have been screened. Peptides that bound specifically to CD40 C terminus were identified by secondary screens against empty vector or irrelevant bait proteins to eliminate false positives. Each of the positive clones we isolated contained the full-length TRAF2 open reading frame. To further confirm the result, TRAF2 with testing baits, empty vector, and control vector (human lamin C) were then co-transformed into yeast and restreaked into the most stringent plates (SD-Ade, -His, -Leu, and -Trp). After 48 h of incubation, whereas yeast transformed with TRAF2 and an empty vector or human lamin C did not grow, yeast transformed with TRAF2 and the C terminus of CD40 (Del260) grew almost as well as yeast transformed with the TRAF2 and full-length tail of CD40 cytoplasmic domain (Fig. 4A). To quantitatively compare the strength of these interactions, we measured β-galactosidase activity in the same strains. Yeast expressing TRAF2/Del260 displayed β-galactosidase activity that was comparable with TRAF2/ΔT2,3,6 and slightly lower than TRAF2/wild type (Fig. 4B). Taken together, results from yeast two-hybrid studies indicated that the C terminus of CD40 by itself could interact with TRAF2.

**DISCUSSION**

TRAFs have been shown to be the essential components of CD40-mediated B cell proliferation, molecular cell surface up-regulation, germinal center formation, generation of memory B cells, isotype switching, affinity maturation, and other events necessary for efficient thymus-dependent humoral immunity (1, 13). In this study, we used site-directed mutagenesis assays and yeast two-hybrid screening to identify a new functional domain that contains a TRAF2 binding motif in the highly conserved C terminus of the CD40 cytoplasmic tail.

The sequence of human and murine CD40 C terminus is completely conserved, except for the fact that the murine CD40 has an extended 11-amino acid tail. We determined that this 11-amino acid extension was not important to the up-regulation of CD23 or growth arrest (data not shown). A previous study showed that with the intact TRAF2/3 (PVQET) binding site, deleting the C terminus of CD40 cytoplasmic tail reduced TRAF2 binding to $-40\%$ of control level (4). Other reports have...
shown that the C terminus CD40 cytoplasmic tail may also
affect TRAF2 binding suggesting the role of CD40 C terminus
in stabilizing canonical TRAF binding at PVQET motif (11, 12).
Our results have extended these observations by finding a
second independent TRAF2 binding site in CD40 C terminus.
We have shown that, with deletion of all the canonical TRAF
binding sites, signaling through this newly identified TRAF2
binding site was able to up-regulate CD23, which functions as
a regulatory receptor for B cells. Finally, this C-terminal do-
main can transduce growth arrest upon CD40 engagement.

Previous studies have shown two independent domains of
CD30 cytoplasmic tail that interact with TRAF2 (Fig. 5) (15,
16). Crystallographic analysis of receptor recognition of TRAF2
has further identified that both PXQXT and SXXE motifs are
recognized by TRAF2 (17, 18). In fact, they all fit into the major
TRAF2 binding consensus sequence, (P/S/A/T/)(Q/E/E) (19).
CD40, like CD30, contains both of the PXQXT and SXXE motifs
in the cytoplasmic tail (Fig. 5). The failure of detection of
TRAF2 binding in the CD40 peptide containing the SXXE motif
in a previous study may have been because of alterations in the
surrounding amino acid environment (16). Indeed, in our ex-
periments, M12 cells expressing chimeric CD40, which has
only 9 amino acids including the SXXE motif in the cytoplas-
mic tail (270–278), showed no activities similar to the negative
control M12/STOP222 (data not shown).

The finding of a second TRAF2 binding site in the CD40
cytoplasmic tail raises numerous questions. First, what is the
physiological role of the second TRAF2 binding site in naive B
cell activation? B cells from human/mouse CD40 transgenic
mice that lack the canonical TRAF binding sites (ΔT2,3,6) are
normal with regard to CD40-mediated NFκB activation, B cell
proliferation, up-regulation of surface molecules, and early Ig
production (1). This suggests that this new TRAF2 site may be
critical for many of these early activities. Mice expressing only
the C-terminal TRAF2 site are being produced to address these
issues. However, another report has shown (using similarly
engineered mice) that the ΔT2,3,6 mice were defective in early B
cell responses but could isotype-switch to IgG2b (13). Al-
though the explanation for such divergent results is unclear at
this time, one possible explanation is that, in our studies,
trimmerized recombinant CD40 ligand was used for CD40 stim-
ulation, whereas in the latter studies, anti-CD40 antibodies
were used. A previous report has shown that forming a trimeric
CD40 ligand and CD40-receptor complex is important for its
signaling (20). Weak stimulation from using anti-CD40 anti-
odies or supernatants of CD40L transfected cells in other
reports might result in the failure of detecting those functional
activities from the second TRAF2 binding site. Differences in
the in vivo responses from these studies can be readily ac-
counted for by the fact that the CD40 transgene was expressed
using the class II major histocompatibility complex promoter in
one set of studies (1) and the Ig promoter in others (13). It is our
opinion that expression of the CD40 transgene under the class
II major histocompatibility complex promoter is preferred be-
cause it replaces CD40 in most of the physiologically relevant
cells of the immune system. Furthermore, differences in ex-
pression levels of transgene could also contribute to the dispar-
ities in the reported experimental results. A second question

**Fig. 3.** Association of TRAF2 with the CD40 mutants. M12 cells expressing different chimeric CD40 molecules were
biotinylated and then stimulated with recombinant hCD154 for 15 min. Cell lysates were then subjected to immuno-
precipitation with anti-CD40 monoclonal antibody followed by blotting with anti-
TRAF2. For chimeric CD40 blotting, the membrane was stripped and reprobed
with anti-biotin antibody. Similar results

**Fig. 4.** Interaction test of TRAF2 with different cytoplasmic tail mutants of CD40 using yeast two-hybrid assay. A, TRAF2 with
testing baits, empty vector, and control vector (human lamin C) were co-transformed into yeast. The interaction phenotype was estimated by the
physiological role of the second TRAF2 binding site in naı¨ve B
cell activation? B cells from human/mouse CD40 transgenic
mice that lack the canonical TRAF binding sites (ΔT2,3,6) are
normal with regard to CD40-mediated NFκB activation, B cell
proliferation, up-regulation of surface molecules, and early Ig
production (1). This suggests that this new TRAF2 site may be
critical for many of these early activities. Mice expressing only
the C-terminal TRAF2 site are being produced to address these
issues. However, another report has shown (using similarly
engineered mice) that the ΔT2,3,6 mice were defective in early B
cell responses but could isotype-switch to IgG2b (13). Al-
though the explanation for such divergent results is unclear at
this time, one possible explanation is that, in our studies,
trimmerized recombinant CD40 ligand was used for CD40 stim-
ulation, whereas in the latter studies, anti-CD40 antibodies
were used. A previous report has shown that forming a trimeric
CD40 ligand and CD40-receptor complex is important for its
signaling (20). Weak stimulation from using anti-CD40 anti-
odies or supernatants of CD40L transfected cells in other
reports might result in the failure of detecting those functional
activities from the second TRAF2 binding site. Differences in
the in vivo responses from these studies can be readily ac-
counted for by the fact that the CD40 transgene was expressed
using the class II major histocompatibility complex promoter in
one set of studies (1) and the Ig promoter in others (13). It is our
opinion that expression of the CD40 transgene under the class
II major histocompatibility complex promoter is preferred be-
cause it replaces CD40 in most of the physiologically relevant
cells of the immune system. Furthermore, differences in ex-
pression levels of transgene could also contribute to the dispar-
ities in the reported experimental results. A second question

**Fig. 5.** Alignment of the TRAF2 binding sites of CD40 with
CD30. Two TRAF2 consensus binding domains are shown in the cyto-
plasmic tail of CD40 and CD30. Amino acid residues are boxed if they
are identical.
that emerges is whether cooperativity between the two TRAF2 sites is critical in recruiting TRAF complexes or whether these sites operate independently (15). As we study the immunological capacities of mice engineered to express one or two TRAF2 sites, the physiological role of the two TRAF2 binding sites in CD40 tail should become apparent. Even though TRAF2 interacts with several TNFR superfamily members, it may function in different ways depending on the site within the receptor to which it binds (21). Different timing, the amount of TRAFs, or different interaction proteins may all contribute to the ability of TRAFs to transduce specific biologic signals.

In summary, we have identified a new functional domain in the C terminus of CD40 cytoplasmic tail that can recruit TRAF2 upon CD40 engagement. The physiological role of the second TRAF2 binding site and how these binding sites interact in concert within the complicated network of CD40 signaling remains to be further investigated.

Acknowledgments—We thank Cory Ahonen and Dr. Arti Gaur for helpful advice.

REFERENCES

1. Ahonen, C., Manning, E., Erickson, L. D., O’Connor, B. P., Lind, E. F., Pullen, S. S., Kehry, M. R., and Noelle, R. J. (2002) Nat. Immunol. 3, 451–456
2. Manning, E., Pullen, S. S., Souza, D. J., Kehry, M., and Noelle, R. J. (2002) Eur. J. Immunol. 32, 39–49
3. Foy, T. M., Aruffo, A., Bajorath, J., Buhlmann, J. E., and Noelle, R. J. (1996) Annu. Rev. Immunol. 14, 591–617
4. Lee, E., Welsh, K., Matsuzawa, S., Zapata, J. M., Kitada, S., Mitchell, R. S., Ely, K. R., and Reed, J. C. (1999) J. Biol. Chem. 274, 22414–22422
5. Pullen, S. S., Miller, H. G., Everdeen, D. S., Dang, T. T., Crute, J. J., and Kehry, M. R. (1998) Biochemistry 37, 11836–11845
6. Pullen, S. S., Dang, T. T., Crute, J. J., and Kehry, M. R. (1999) J. Biol. Chem. 274, 14246–14254
7. Berberich, I., Shu, G. L., and Clark, E. A. (1994) J. Immunol. 153, 4357–4366
8. Berberich, I., Shu, G., Siebelt, F., Woodgett, J. R., Kyriakis, J. M., and Clark, E. A. (1996) EMBO J. 15, 92–101
9. Li, Y. Y., Baccam, M., Waters, S. B., Pessin, J. E., Bishop, G. A., and Koretzky, G. A. (1996) J. Immunol. 157, 1440–1447
10. Zhu, N., Ramirez, I. M., Lee, R. L., Magnuson, N. S., Bishop, G. A., and Gold, M. R. (2002) J. Immunol. 168, 744–754
11. Hostager, B. S., and Bishop, G. A. (1999) J. Immunol. 162, 6307–6311
12. Haxhinasto, S., Hostager, B. S., and Bishop, G. A. (2002) Immunity 17, 1145–1149
13. Jabara, H., Lacovina, D., Tsitsikov, E., Mizoguchi, E., Bhan, A., Castiglione, E., Dedeglu, F., Pivniouk, V., Brodeur, S., and Geha, R. (2002) Immunity 17, 265–276
14. Golstein, M. D., and Watts, T. H. (1998) J. Immunol. 157, 2837–2843
15. Lee, S. Y., Kandala, G., Liu, M. L., Liu, H. C., and Chiu, Y. (1998) Proc. Natl. Acad. Sci. U. S. A. 93, 9699–9703
16. Boucher, L. M., Marenegere, L. E., Lu, Y., Thukral, S., and Mak, T. W. (1997) Biochem. Biophys. Res. Commun. 233, 592–600
17. Park, Y. C., Burkit, V., Villa, A. R., Tong, L., and Wu, H. (1999) Nature 398, 533–538
18. McWhirter, S. M., Pullen, S. S., Holton, J. M., Crute, J. J., Kehry, M. R., and Alber, T. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 8408–8413
19. Ye, H., Park, Y. C., Kreishman, M., Kieff, E., and Wu, H. (1999) Mol. Cell 4, 321–330
20. Werneburg, B. G., Zoog, S. J., Dang, T. T., Kehry, M. R., and Crute, J. J. (2001) J. Biol. Chem. 276, 43334–43342
21. Hostager, B. S., and Bishop, G. A. (2002) J. Immunol. 168, 3318–3322
CD40 Signaling through a Newly Identified Tumor Necrosis Factor Receptor-associated Factor 2 (TRAF2) Binding Site
Li-Fan Lu, W. James Cook, Ling-Li Lin and Randolph J. Noelle

J. Biol. Chem. 2003, 278:45414-45418. doi: 10.1074/jbc.M309601200 originally published online September 5, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M309601200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 21 references, 12 of which can be accessed free at http://www.jbc.org/content/278/46/45414.full.html#ref-list-1