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Role of TNF Receptor-Associated Factor 2 in the Activation of IgM Secretion by CD40 and CD120b

Bruce S. Hostager* and Gail A. Bishop 2*†‡

TNFR-associated factors (TRAFs) participate in the signaling of many TNFR family members, including CD40, CD120a (TNFR1), and CD120b (TNFR2). Previously, we found that a dominant-negative TRAF2 molecule inhibits CD40-mediated Ab secretion by the mouse B cell line CH12.LX. However, disruption of the TRAF2 binding site in the cytoplasmic domain of CD40 does not diminish the ability of CD40 to stimulate Ab secretion, nor is this mutation able to circumvent the inhibition of Ab secretion by dominant-negative TRAF2. Here we demonstrate that CD40-induced TNF stimulates IgM production through CD120b and that CD120b signaling is required for optimal CD40-induced IgM secretion. Furthermore, although both CD40 and CD120b can bind TRAF2, TRAF2-dependent CD40 signals cannot substitute for TRAF2-dependent CD120b signals in the activation of IgM secretion. Our results indicate a potentially important role for CD120b in the activation of IgM secretion and that TRAF2 is used by CD40 and CD120b in distinct ways. The Journal of Immunology, 2002, 168: 3318–3322.

Signals from CD40 are critical to the activation of APCs during T-dependent immune responses (1). In B lymphocytes, engagement of CD40 by its ligand (expressed on activated T lymphocytes) triggers signaling events that contribute to cell proliferation, Ig secretion, isotype switching, and the humoral memory response. Some of the earliest molecular events in signaling include the localization of CD40 to cholesterol-enriched membrane microdomains and the recruitment of TNFR-associated factors (TRAFs)3 to the cytoplasmic domain of CD40 (2). Singly or in concert, TRAFs 2, 3, 6, and potentially 1 and 5, mediate communication between CD40 and signaling intermediates that in turn trigger the activation of kinases such as c-Jun kinase and transcription factors such as NF-κB (reviewed in Ref. 3).

Previously, we examined the contribution of TRAF2 to the CD40-mediated induction of IgM secretion and found that induced overexpression of a truncated, dominant-negative (DN) mutant TRAF2 inhibits this function by ~50% (4). Interestingly, our earlier structure-function studies of CD40 signaling to B cells had shown that mutations in the CD40 cytoplasmic domain that prevent TRAF2 binding do not decrease CD40-mediated IgM production (5), but DNTraf2 expression also inhibits IgM secretion induced by these CD40 mutants (4). A potential explanation of these findings is that CD40-induced IgM secretion involves at least one other TRAF2-binding member of the TNFR superfamily and expression of DNTraf2 is exerting its effect via this second receptor. We hypothesized that this second receptor might be CD120a and/or CD120b. The present study was designed to test this hypothesis.

We show here that TNF produced by CD40-stimulated B cells triggers signaling to the B cells through CD120b and that this signaling is an important component of CD40-mediated IgM production. Although both CD120b and CD40 interact directly with TRAF2 in B cells, and although direct TRAF2-CD40 binding is required for other CD40 functions (3), activation of IgM secretion by CD40 does not require CD40-TRAF2 binding. In contrast, CD120b-mediated IgM secretion requires TRAF2, and this TRAF2 signal cannot be provided by CD40. Our results thus demonstrate that specific TRAF functions in particular cell types can be markedly influenced by the receptor signaling complexes with which the TRAF interacts.

Materials and Methods

Cells

The mouse B cell line CH12.LX and transfectants expressing human CD40 (hCD40) molecules and isopropylthio-β-d-galactoside (IPTG)-inducible DNTraf2 (aa 87–501 of mouse TRAF2 (mTRAF2) with an amino-terminal FLAG tag) have been described (4). The mouse B cell line M12.4.1 has been previously described (6). J774 cells were from the American Type Culture Collection (Manassas, VA). T-depleted mouse C57BL/6 splenocytes were prepared as previously described (7). In some experiments, small, high-density (resting) B lymphocytes were purified from the splenocytes using Percoll density gradient centrifugation. All mouse cells were maintained in RPMI 1640 containing 10% FCS (HyClone, Logan, UT), 10 μM 2-ME, and antibiotics. In cells stably transfected with inducible DNTraf2, induction was accomplished by incubating the cells with 100 μM IPTG for 15 h before stimulation of the cells through CD40. S9 insect cells and S9 cells expressing mCD154 have been described previously (7, 8).

DNA constructs

A cDNA construct coding for a chimeric hCD40-CD120b molecule was prepared using overlap extension PCR (9). The template for the external hCD40 domain was a previously reported hCD40 expression plasmid (5). The cDNA prepared from the mouse macrophage cell line J774 was used as template for the transmembrane and cytoplasmic portions of CD120b. Sequences of the PCR primers used in making the hCD40-CD120b hybrid were as follows: 5′-agtctggaccgctctctgggagcc-3′, 5′-caattggaagagatgc-3′, 5′-cttttagatctgagggctctctgggagcc-3′, and 5′-ttttctgttttggagggctctctgggagcc-3′. The completed PCR products were ligated into the mammalian expression vector pR5.5(neo) (10).
**Detection of CD120a and CD120b mRNA**

RNA was isolated from 5 × 10^6 CH12.LX, M12.4.1, or J774 cells using an RNA extraction kit (RNeasy; Qiagen, Valencia, CA). The cDNA was generated from the RNA using reverse transcriptase (Promega, Madison, WI) and a poly(T) oligonucleotide primer. The cDNA was then amplified by PCR using primers specific for the transmembrane and cytoplasmic domains of CD120a (5'-gttgtctgtgcccagactagtct-3'; 5'-aatttagaattcaggtggtct-3'), CD120b (5'-cccaagcagctgtggtgctctct-3'), GAPDH (as a positive control) (18).

**Immunoprecipitations**

CH12.LX cells (1 × 10^7) stably transfected with hCD40 or hCD40-CD120b were stimulated in 1 ml of culture medium for 15 min (37°C) with 10 μg of anti-hCD40 to induce association of TRAF molecules with the receptors. The cells were pelleted by centrifugation, resuspended in 400 μl of lysis buffer (60 mM n-octyl β-D-glucopyranoside, 1% Triton X-100, 0.1% SDS, 20 mM Tris (pH 7.5), 150 mM NaCl, 50 mM β-glycerophosphate, 50 μg/ml PMSF, 50 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A), and sonicated (on ice) with a probe sonicator (Branson Ultrasonics, Danbury, CT) to reduce viscosity. After a 15-min incubation on ice, insoluble debris was pelleted by centrifugation and discarded. Protein G-Sepharose beads (10 μl of a 50% suspension) were added to the supernatant, which was then incubated on a rotating mixer at 4°C for 2 h. The beads were washed several times with lysis buffer containing 400 mM NaCl and then analyzed by SDS-PAGE and Western blotting. Peroxidase-labeled Abs were visualized on Western blots using a chemiluminescent detection reagent (Pierce, Rockford, IL).

**Results**

In previous work, we characterized features of the cytoplasmic domain of CD40 that contribute to signaling in B lymphocytes (5, 19). To accomplish this, we designed a variety of human CD40 molecules containing cytoplasmic domain mutations and examined the functional activities of these molecules in stably transfected mouse B lymphocyte lines. The cytoplasmic domains of mouse and hCD40 are highly homologous, and they signal similarly in mouse B cells (5, 19). Species-specific anti-CD40 mAbs allowed us to compare the function of the hCD40 mutants with that of the endogenous mCD40. We determined that hCD40ΔΔ22, containing a 22-aa cytoplasmic truncation, retains a wild-type ability to stimulate IgM secretion in the mouse B cell line CH12.LX. Interestingly, unlike full-length hCD40, hCD40ΔΔ22 binds TRAF2 only weakly, if at all (4). TRAF2 is one member of a family of intracellular proteins that appear to function as adapter molecules, linking TNFR family members to downstream signaling pathways. Although TRAF2 has been implicated in the CD40-mediated activation of NF-κB and c-Jun NH2-terminal kinase (JNK) (20), our results with hCD40ΔΔ22 indicate that a direct interaction between CD40 and TRAF2 is not required for activation of IgM secretion by CD40. However, in cell lines stably transfected with inducible TRAF2 or DNTRAF2, overexpression of the former augments CD40-mediated IgM secretion, whereas expression of the latter attenuates IgM production (4). These effects are observed even if cells are stimulated through hCD40ΔΔ22, suggesting that another TRAF2-binding receptor contributes to the IgM secretion initiated by CD40 signaling.

Because TNF has been shown to promote Ab production by B cells in vitro (21) and in vivo (22) and because TRAF2 interacts with both TNFRs, we considered the possibility that one or both of these receptors is responsible for the augmentation of CD40-mediated IgM secretion. Stimulation of CH12.LX cells or small resting splenic B lymphocytes with CD154-expressing insect cells resulted in the rapid production of TNF (Fig. 1). Consistent with the hypothesis that one of the TNFRs augments CD40-stimulated Ab secretion, stimulation of CH12.LX cells with a saturating amount of exogenous TNF activated IgM secretion to approximately one-half the level induced by CD40 engagement (Fig. 2A). To confirm
that the amount of TNF produced in response to CD40 stimulation is sufficient to augment IgM secretion, we also examined IgM production by CD40-stimulated CH12.LX cells in the presence or absence of neutralizing anti-TNF Abs. Anti-TNF Abs reduced by \( \sim 50\% \) the number of Ab-secreting cells that otherwise develop as the result of CD40 stimulation (Fig. 2A). Similarly, anti-TNF or the soluble TNFR drug Enbrel inhibited CD40-stimulated IgM secretion by T cell-depleted mouse splenocytes (Fig. 2).

Potentially, one or both TNFRs (CD120a and CD120b) contribute to Ab secretion. Because both receptors have been shown to interact with TRAF2, signaling from either receptor could potentially be disrupted by DOTRAF2. To determine which receptor is involved in TNF-induced IgM secretion, we examined CH12.LX cells for TNFR expression. Although we were unable to detect cell surface TNFR expression by flow cytometry using commercially available Abs (data not shown), CD120b gene expression in CH12.LX cells was readily detected by RT-PCR (Fig. 3). CD120a gene expression, although detected in the mouse macrophage cell line J774, was not detected in CH12.LX cells or in a second mouse B cell line, M12.4.1. Consistent with these findings, unstimulated human B cells have been reported to express CD120b, but little or no CD120a (23, 24). Stimulation of CH12.LX or M12.4.1 cells through CD40 for 2 or 6 h, respectively, did not result in detectable up-regulation of CD120a mRNA (data not shown).

To confirm that the CD120b on B lymphocytes has the ability to induce IgM secretion, we examined the functional activity of a hybrid molecule consisting of the extracellular domain of CD40 fused to the transmembrane and intracellular domains of CD120b. As previously demonstrated for CD120b (25), the hCD40-CD120b hybrid was able to bind TRAF2 (Fig. 4A). Interestingly, the co-precipitation of TRAF2 with the hybrid appeared to be more efficient than with hCD40. Stimulation of stably transfected CH12.LX cells through the hCD40-CD120b hybrid resulted in levels of Ab secretion similar to that achieved using rTNF as a stimulus (Fig. 4B). A similar hybrid molecule with the cytoplasmic domain of CD120a did not induce IgM secretion when stably expressed at similar levels on CH12.LX cells (data not shown). Furthermore, we found that the CD40-mediated activation of IgM secretion by freshly isolated mouse splenocytes was inhibited by a neutralizing anti-CD120b mAb, but not by a similar anti-CD120a mAb (Fig. 4C).

Based on the ability of CD120b to activate Ab secretion and on the observation that DOTRAF2 inhibits the activation of IgM secretion by a truncated CD40 mutant (which is unable to bind TRAF2) (4), we proposed that TRAF2 is important for CD120b but not CD40-mediated IgM secretion. To test this possibility more directly, B cells stably transfected with an inducible DOTRAF2 construct were stimulated through CD40 or TNF in the presence or absence of neutralizing anti-TNF Abs and then assayed for TNF production and Ab secretion. DOTRAF2 did not significantly inhibit TNF production by CD40-stimulated cells (Fig. 5A), but did inhibit Ab secretion by \( \sim 50\% \) (Fig. 5, B and C). Anti-TNF inhibited IgM secretion to a similar extent but, significantly, did not enhance the inhibition mediated by DOTRAF2 (Fig. 5B). DOTRAF2 induction almost completely abrogated TNF-induced IgM production (Fig. 5C). Together, these observations indicate

\[ \text{FIGURE 1. CD40 stimulates TNF production by B lymphocytes. CH12.LX or small resting B lymphocytes were stimulated with 25 pg/ml TNF (predetermined to be saturating concentration) or 2 \mu g/ml anti-mCD154 (■) or S9 cells infected with a baculovirus encoding mCD154 (○). TNF production was measured by ELISA; similar results were obtained in a second experiment. Results are presented as the mean ± SE of triplicate samples.} \]

\[ \text{FIGURE 2. TNF participates in CD40-mediated IgM secretion. A, CH12.LX cells were stimulated with 25 pg/ml TNF (predetermined to be a saturating concentration) or 2 \mu g/ml anti-mCD40 mAb for 72 h in the presence (■) or absence (○) of 10 \mu g/ml anti-TNF mAbs. After stimulation, viable cells were counted, and IgM secretion was assessed using a hemolytic plaque assay. The number of plaque-forming cells per million viable cells is shown on the y-axis. Results are presented as the mean ± SE of replicate samples. Similar results were obtained in two additional experiments.} \]

\[ \text{FIGURE 3. Expression of mRNA coding for CD120a and CD120b in mouse cell lines. Using RT-PCR, expression of CD120a and CD120b was assessed in the mouse B cell lines CH12.LX and M12.4.1 and the macrophage cell line J774. Samples of the PCRs were visualized on agarose electrophoretic gels stained with ethidium bromide. Parallel PCRs were performed with GAPDH primers as a positive control. Similar results were obtained in a second experiment.} \]
that anti-TNF and DNTRAF2 block the same step in the activation of IgM secretion: CD120b signaling.

Discussion
Several TNFR family members, including CD40 and both TNFRs, have been shown to interact with TRAF2, suggesting that each of these receptors engages at least one common downstream signaling pathway. Our results demonstrate, however, that TRAF2 may function in different capacities depending on the receptor with which it associates. Whereas CD120b-mediated IgM secretion is dependent on TRAF2, IgM secretion mediated by CD40 is not. Furthermore, it appears that binding of TRAF2 by CD40 cannot substitute for the TRAF2-dependent signal generated by CD120b. Were this the case, the inhibition of CD40-stimulated IgM secretion by anti-TNF should be augmented by DNTRAF2 expression. It is not (Fig. 5).

There are likely to be a number of factors that contribute to the ability of TRAFs, as illustrated here, to perform differently when associated with distinct receptors. First, each well-characterized member of the rapidly growing TNFR superfamily binds to TRAF molecules, but each binds a distinct and not completely overlapping set of TRAFs. Thus, CD40 in B cells is reproducibly found to bind TRAFs 2, 3, and 6 directly and TRAF1 via heterodimerization with TRAF2. Binding to TRAF5 has been shown by two-hybrid yeast analysis and transient overexpression in transformed epithelial cells, but this has not yet been confirmed in B cells (3). CD120b also binds TRAF2 and TRAF1, but it does not associate with TRAFs 3, 5, or 6 (20). It seems a reasonable prediction that the presence of additional TRAFs in a signaling complex influences the ultimate function of each TRAF. For example, because TRAF3 binds to CD40 at a site largely overlapping that of TRAF2 and has been implicated as a potential negative regulator of signaling in B cells (4), it is quite likely that the close proximity of TRAF3 in a receptor complex affects TRAF2 function. Another difference between individual receptor-TRAF interactions is their avidity. TRAF2 and TRAF3 both bind robustly to CD40 in B cells, but the association of TRAF2 with a virally encoded transforming protein that mimics CD40, LMP1, is much weaker. Interestingly,
association with CD40 stimulates degradation of TRAF2, but asso-
ciation with LMP1 expressed in the same B cells does not, a
difference that may be explained in part by weaker binding and
which may contribute to the amplified signaling and transforming
properties of the viral protein (26). It is also likely that TRAFs
exhibit distinct functional properties in distinct cell types, even
when binding to the same receptor.

The TRAF2-dependent signaling pathway activated by CD120b
remains to be identified. Previous work indicates that TRAF2 is
potentially involved in activating both JNK and NF-κB (3). How-
ever, both of these signals are supplied to CH12.LX cells by CD40
engagement (19, 27), and because CD40 signaling cannot substi-
tute for CD120b signals in our experiments, it is unlikely that the
critical CD120b signal is mediated by either JNK or NF-κB. In
addition, we have been unable to detect significant activation of
either JNK or NF-κB in CH12.LX cells stimulated with TNF or
through engagement of hCD40-CD120b (data not shown). To-
gether, our results demonstrate that the TNFR family member dic-
tates the signaling pathway with which TRAF2 associates. The
receptor may accomplish this by inducing subtle changes in
TRA2F conformation or through the recruitment of additional pro-
teins that interact with TRAF2 in the signaling complex.

Our results also support the possibility that CD120b contributes
to the activation of humoral immune responses, because neutral-
izing Abs to TNF markedly reduced CD40-mediated IgM produc-
tion by both normal splenic B cells and CH12.LX. Similar results
have been reported in human B cells (21). However, it has also been
reported that CD120b-deficient mice do not show a substan-
tial defect in the IgM response to SRBCs (28); responses to other
Ags have not been reported. This shows that, at least in some
situations, IgM responses are not highly dependent upon CD120b
signaling. However, it is possible that the absence of CD120b from
the earliest stages of embryogenesis can be compensated by ad-
justments to other signaling pathways, because the redundancy of
the mammalian immune system is well documented. Additionally,
the immune response to the model Ag SRBC may not be repre-
sentative of IgM responses to all T-dependent Ags. In this regard,
it is interesting to note that CD120b－/－ mice have much lower
survival rates than wild-type mice after infection with ectromelia
virus (29). Although the basis of this increased mortality remains
to be determined, it would be of interest to examine IgM responses
to virus in these mice.

In summary, we have shown that TRAF molecules can function
in CD40 signaling to B cells, not only by direct binding to CD40,
but also by association with other receptors that participate in
CD40-mediated effects. We believe that this concept is very likely
to apply to additional TRAF-receptor interactions and illustrates
the multiple ways in which this family of cytoplasmic adapter
proteins can regulate signaling.

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