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Cutting Edge: Molecular Mechanisms of Synergy Between CD40 and the B Cell Antigen Receptor: Role for TNF Receptor-Associated Factor 2 in Receptor Interaction

Sokol A. Haxhinasto,* Bruce S. Hostager,† and Gail A. Bishop‡

Optimal Ag-specific B lymphocyte activation requires both recognition of Ag by the B cell Ag receptor (BCR) and contact-mediated interactions with Ag-specific Th lymphocytes. One of these interactions involves ligation of B cell CD40 by T cell-expressed CD154. CD40 signaling is crucial for Ab production, isotype switching, up-regulation of surface molecules, development of germinal centers, and the humoral memory response. The signaling pathways emanating from the BCR and CD40 are able to cooperate, but the molecular mechanisms responsible for this interaction are incompletely understood. The present study explored the roles of signaling motifs in the CD40 cytoplasmic tail in this synergy. We find that threonine in the PXQXT motif in the TNFR-associated factor-2 binding site is critical for synergistic effects of CD40 and BCR signals, independent of its phosphorylation. Furthermore, data suggest an indirect role for TNFR-associated factor-2 in the cooperative signaling. The Journal of Immunology, 2002, 169: 1145–1149.

The B cell Ag receptor (BCR) plays a critical role in B cell development and activation. Early in B cell development BCR expression is necessary for allelic exclusion and gene recombination at the Ig L chain loci, while in immature B cells the BCR is important for tolerance induction and selection of B cells into the peripheral pool. In mature peripheral B cells the BCR contributes to Ag-dependent activation and selection of memory B cells. Engagement of the BCR by Ag activates signaling cascades that ultimately result in the transcription of genes associated with B lymphocyte activation. However, BCR engagement alone is usually not sufficient to induce proliferation and differentiation of B cells. Full activation requires contact-mediated interaction with Ag-specific Th cells and engagement of additional signaling molecules, including CD40 (3, 4).

CD40 is a member of the TNFR family that plays an important role in B lymphocyte activation. CD40 engagement by its ligand, CD154 expressed on Th cells, stimulates B cell proliferation, differentiation, isotype switching, up-regulation of surface molecules, development of germinal centers, and immunological memory (5). CD40 does not possess intrinsic kinase activity and there are no conserved tyrosine residues in the cytoplasmic (CY) tail between human CD40 (hCD40) and mouse CD40 (mCD40). However, the CY tail of CD40 does possess several potentially phosphorylatable serine (three in human and two in mouse) and threonine (four in human and three in mouse) residues. Several members of the TNFR-associated factor (TRAF) family have been shown to bind to CD40 and appear to serve as adapter proteins in the CD40 signaling pathway (6). However, the exact role of TRAFs in CD40 signaling is still unresolved.

The signaling pathways emanating from the BCR and CD40 are able to cooperate in a synergistic or additive manner (7, 8). Despite obvious structural differences between the two receptors, BCR and CD40 have both been reported to stimulate kinases such as extracellular signal-regulated kinase, c-Jun NH2-terminal kinase, p38, and ultimately similar transcription factors, such as NF-κB, NF-AT, and activating protein 1 (9, 10). Regardless of similarities in their signaling pathways, there are distinct signaling mechanisms unique to each receptor that may contribute to the cooperative/synergistic nature of pathways initiated by these receptors. The molecular mechanisms of interaction between BCR and CD40 are not completely understood. We have investigated the role of structural motifs in the CD40 CY tail in the synergy between BCR and CD40. Interestingly, while a single threonine in the context of the full-length tail was found to be both necessary and sufficient to allow synergy, this was independent of its phosphorylation status. Our findings also demonstrate a differential role for TRAF2 and TRAF3 in BCR-CD40 synergy. TRAF2 appears to be required for the synergy between BCR and CD40 if TRAF3 is able to bind to CD40. However, the major function of TRAF2 in this context may be to counteract a negative effect of TRAF3.

Materials and Methods

Cells and transfections

The mouse B cell line CH12.LX expresses surface IgM specific for phosphatidylcholine, an Ag found on the surface of SRBC (11), and has been described previously (12). Cells were cultured in RPMI 1640 supplemented
with 10% FCS, 10 μM 2-ME, and antibiotics (BCMC-10). Spodoptera frugiperda (Sf9) cells infected with wild type, a recombinant baculovirus expressing mCD154, or Chinese hamster ovary cells expressing mCD154 were prepared as previously described (7, 13). C57BL/6 mouse splenic B cells were prepared as described (7). Stable transfection of cells with hCD40 constructs was performed using electroporation (14). G418-resistant clones were analyzed for surface expression of hCD40 using FACS. Transfectants with similar levels of expression of hCD40 were selected for experiments.

**Antibodies**

Mouse IgG1 isotype control mAb (MOPC-21) was purchased from Sigma-Aldrich (St. Louis, MO). Rabbit anti-TRAF3 (H-122) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-TRAF2 was purchased from MBL (Nagoya, Japan). Goat anti-rabbit HRP was purchased from Bio-Rad (Hercules, CA). The following Abs were produced in our laboratory by hybridomas purchased from the American Type Culture Collection (Manassas, VA) or were gifts of the indicated individuals: anti-hCD40 (G28-5, mIgG1), anti-mCD40 (1C10, rat IgG2a; from Dr. F. Lund, Trudeau Institute, Saranac Lake, NY), anti-mouse IgE (EM95.3, isotype control, rat IgG2a; from Dr. T. Waldschmidt, University of Iowa, Iowa City, IA). Goat anti-mouse μ-chain-specific F(ab’)_2 was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

**Assays for B cell function**

**IgM secretion assay.** The IgM secretion assay has been described previously (15). Briefly, CH12.LX and its transfected subclones were cultured in 96-well plates (1.5 × 10^3/well) with various stimuli. Anti-CD40 Abs were used at a final concentration of 2 μg/ml SRBC (Elmira Biologicals, Iowa City, IA) at a final concentration of 0.1% were used as Ag. Triplicate cultures were incubated for 72 h, then viable cells were counted by trypan blue exclusion. IgM-secreting cells were enumerated as SRBC hemolytic plaques (16).

**Cytokine ELISAs.** For IL-6, CH12.LX cells or T-depleted splenic B cells (1 × 10^6) plus mCD154-expressing cells were cocultured at a ratio of B cells:mCD154-expressing cells of 4:1 for 48 h, and the supernatants were tested for IL-6 by ELISA as previously described (13). Cells were stimulated with 10 μg/ml goat anti-mouse μ-chain-specific F(ab’)_2 (Jackson ImmunoResearch Laboratories). For TNF-α, B cells (1 × 10^6) splenocytes or 5 × 10^6 CH12.LX cells per well) were resuspended in 200 μl of BCM-10 with various stimuli in an anti-TNF-coated 96-well flat-bottom plate. Cells were stimulated with 10 μg/ml anti-IgM Ab with or without 5 × 10^6 mCD154-expressing cells for 3 h. Splenocytes were stimulated with 2.5 × 10^4 mCD154-expressing cells for 4 h. Culture supernatants were assayed for TNF-α as described (17).

**Immunoprecipitation and Western blotting**

CH12.LX cells (10^5) transfected with different hCD40 constructs were stimulated for 15 min with S99-hCD154 cells (2.5 × 10^6). Both CD40 and TRAIL receptors are recruited to detergent-insoluble membrane rafts upon CD40 engagement (18). Cells were lysed and the detergent-soluble and -insoluble fractions were separated by centrifugation as described (18). The detergent-insoluble pellet was resuspended in octylglucopyranoside buffer and hCD40 was immunoprecipitated as described (17). Immunoprecipitated proteins were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes. A chemiluminescent substrate (Pierce, Rockford, IL) was used to detect HRP-labeled Abs on Western blots.

**Preparation of 32P-labeled cell lysates**

CH12.hCD40 cells (10^7 cells) were cultured in phosphate-free RPMI 1640 medium containing 10% dialyzed FCS (Sigma-Aldrich) and [32P]orthophosphate (Amersham Pharmacia Biotech, Piscataway, NJ) to a final concentration of 1 mCi [32P]P2 ml/10^6 cells for 2 h. Stimuli (10 μg/ml goat anti-mouse μ-chain-specific F(ab’)_2; or isotype control goat Ab fragments, 2.5 × 10^6 S99-hCD154 or S99-Wt vector cells, or 100 ng/ml PMA) were added during the last 1–30 min of incubation. At 5-min intervals, cells were lysed in 1% Nonidet P-40 lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris (pH 7.5), 0.02% NaN3, and protease and phosphatase inhibitors) for 30 min. The supernatants were preclarified for 1.5 h at 4°C with protein G beads preconjugated with isotype control Ab (MOPC-21). The preclarified lysates were immunoprecipitated with protein G beads preconjugated with anti-hCD40 mAb (G28-5). Immunoprecipitated proteins were separated by SDS-PAGE. The gel was dried and autoradiography was performed to detect CD40 (2 days to 2 wk).

**CUTTING EDGE: MECHANISMS OF CD40-BCR SYNERGY**

The mammalian expression vector used for transfection of all hCD40 constructs was pRSV5.neo (19). Oligonucleotide primers containing appropriately positioned point mutations or stop codons were synthesized and used in PCR amplification and subcloning by overlap extension (20). Terminal restriction endonuclease sites were designed into the oligonucleotide primers for inserting the PCR products into the pRSV5.neo vector.

**Results and Discussion**

**BCR and CD40 synergy in B cell activation is evident in multiple cellular events**

It has previously been reported that normal B cells are activated to secrete IgM (21), IL-6 (13), and TNF-α (22) following CD40 stimulation. We have previously shown that the mouse mature B cell line CH12.LX can also be induced to produce IgM, IL-6, and TNF-α following CD40 ligation (13, 14, 17). Data presented in Fig. 1 show that signals from CD40 and BCR can cooperate in stimulating IgM production (7). Thus, CH12.LX is an appropriate model for structure-function studies to examine the molecular basis of BCR-CD40 synergy. While the production of IgM and IL-6 requires several days, production of TNF-α occurs in <3 h. We thus selected both IgM secretion (a late event) and TNF-α production (to allow examination of earlier signaling events) to study BCR-CD40 interaction.

**Threonine 234 is necessary and sufficient for the synergy between BCR and CD40**

Earlier structure-function studies suggested a role for the threonine residue in the PXQXT motif, which is conserved between mCD40 and hCD40 (at position 235 in mouse and 234 in human), in the synergy between BCR and CD40 (14). A hCD40 molecule containing a threonine to alanine substitution at position 234 (T234A) is able to induce IgM secretion similar to wild-type hCD40, but it is not able to synergize with the BCR (14). Signaling through another hCD40 mutant, T234S, mimics wild-type hCD40 (14), suggesting the hypothesis that the presence of a phosphorylatable residue at position 234 is critical for the observed synergy between BCR and CD40. To test this hypothesis, two additional hCD40 mutants were constructed and stably expressed in CH12.LX cells (Fig. 2). The mutant called hCD40NPR contains no phosphorylatable residues (NPR), while hCD40OPR contains T234 as the only phosphorylatable residue (OPR) (Fig. 2). These mutants were...
tested for the ability to cooperate with the BCR in IgM secretion (Fig. 3A) and TNF-α production (Fig. 3B). Both OPR and NPR hCD40 molecules stimulated similar levels of IgM secretion in the absence of BCR signaling (Fig. 3A, hatched bars). However, similar to wild-type hCD40, BCR signaling synergized with hCD40OPR (Fig. 3A, filled bars) (14) but not with the hCD40NPR mutant. Similar findings were observed in TNF-α production (Fig. 3B). Although BCR engagement alone does not stimulate IgM production by CH12.LX cells (12), modest induction of TNF-α by B cells was induced (Fig. 1). To determine whether the level of TNF-α production induced by CD40 plus BCR shows cooperation between the two signals, the calculation described in Fig. 3 was performed on data sets from each transfected subclone. Summary data are shown in Fig. 3B. As indicated, compared with the endogenous mCD40 (which serves as an internal control for the ability of BCR to synergize with CD40 engagement), wild-type hCD40 and OPR CD40 molecules enhanced BCR signaling while T234A and NPR were defective in cooperation. These data demonstrate that, of the potentially phosphorylatable residues in the CD40 CY domain, T234 is both necessary and sufficient for the interaction between BCR and CD40 in IgM secretion and TNF-α production.

Neither BCR nor CD40 signaling affects CD40 phosphorylation

The requirement for T234 suggested that BCR-induced phosphorylation of this residue might be critical for BCR and CD40 to synergize. It has been reported that CD40 is phosphorylated in response to PMA treatment of cells, but the physiological relevance of these findings is unclear (23, 24). To examine the phosphorylation state of the hCD40 CY domain, cells were labeled with [32P]orthophosphate and hCD40 was immunoprecipitated from cells stimulated via BCR, CD40, or both. Currently available anti-mCD40 Abs do not effectively immunoprecipitate mCD40, so CH12.LX expressing wild-type hCD40 was chosen for these experiments because G28-5 mAb works well in immunoprecipitation (17, 25). Although we reproduced the published finding that PMA induces phosphorylation of the CY tail, no constitutive or BCR/CD40-induced phosphorylation of hCD40 was detected at either very early or later time points (Fig. 4). This negative finding was reproducibly seen even at very long exposures of the gels (up to 2 wk).

Differential roles of TRAF2 and TRAF3 in the synergy between BCR and CD40

We considered the possibility that T234 is involved in the recruitment of signaling molecules to the CD40 CY tail, which in turn are important for BCR-CD40 synergy. Indeed, it has been reported that T234 is contained within the binding motif for TRAFs. However, these studies were performed by transient overexpression of these molecules in epithelial cell lines or by in vitro binding studies using GST-CD40 mutants or peptides (26–29). To address the physiologic role of endogenous TRAF-CD40 binding in CD40-BCR synergy, the ability of the different hCD40 mutants to bind endogenous TRAFs in B cells was investigated. Engagement of B cell CD40 is necessary for recruitment of TRAF molecules (18), as is evident with wild-type hCD40 (Fig. 5, first two lanes). In contrast to previous studies performed in artificial systems, we find that the T234A mutant is able to bind TRAF3 in B cells to a similar extent as wild-type hCD40, although it is defective in TRAF3 binding in 293 cells (data not shown). This indicates that B cells have different CD40 binding requirements for TRAFs than epithelial cells, so binding studies in one cell type cannot be fully extrapolated to another. Perhaps an additional uncharacterized cell

### FIGURE 2

Role of threonine 234 in the synergy between BCR and CD40. A, CH12.LX cells stably expressing different hCD40 constructs were tested for IgM secretion. Results are mean ± SE of replicate cultures and represent two similar experiments. B, CH12.LX cells transfected with the wild-type hCD40 molecule were tested for TNF-α production as described in Materials and Methods. The ratio of mCD40 plus BCR to BCR was set to 100% and the (hCD40 + BCR)/BCR is presented as a percentage of the maximum value. Results are mean ± SD of replicate cultures and represent seven similar experiments using two different clones for each hCD40 molecule.

### FIGURE 3

Role of threonine 234 in the synergy between BCR and CD40. A, CH12.LX cells stably expressing wild-type hCD40 were labeled with [32P]orthophosphate and stimulated as indicated by lane number: lane 1, BCR; lanes 2–7, S9-CD154 cells plus anti-IgM for 1, 5, 10, 15, 20, and 30 min, respectively; lane 8, PMA (10 min); lane 9, S9 control cells plus anti-IgM; lane 10, S9-CD154 cells plus isotype control Abs. hCD40 was immunoprecipitated from cell lysates and samples were subjected to SDS-PAGE. The gel was dried and autoradiography was performed to detect CD40 (2-day exposure). B, The same samples were subjected to SDS-PAGE and electrophoresed onto nitrocellulose membranes. Western blotting was performed to confirm similar amounts of hCD40 in each sample.
FIGURE 5. Differential binding of TRAF2 by hCD40 mutants. Cells were stimulated with S9-hCD154 cells for 15 min to induce CD40-TRAF association. Cell lysates were separated into soluble and insoluble fractions and CD40 was immunoprecipitated from the insoluble fraction. Immunoprecipitated proteins were separated by SDS-PAGE and then electroblotted onto nitrocellulose membranes. Arrows indicate the position of specific TRAF bands and a nonspecific band (NS) detected in all samples by the anti-TRAF Abs. A representative experiment of three is shown.

| Table 1. Relationship between TRAF2 and TRAF3 binding and the ability of CD40 to synergize with BCR |
|-----------------|----------|---------|----------|----------|
| **Human CD40**  | Synergy in IgM secretion | Synergy in TNF-α | TRAF2 Binding | TRAF3 Binding |
| Wild type       | ++       | +       | +++      | +++      |
| T234A           |         |         |          |          |
| NPR             | ++       | ++      | -        | ++       |
| OPB             | ++       | ++      | +        | -        |
| Δ22             | +        | +       | -        | -        |

TRA2 (T234A, NPR), were defective. Therefore, TRAF3 binding in the absence of TRAF2 may play a negative role in the synergy between BCR and CD40. Our prior studies and others have suggested a negative role for TRAF3 in signaling by CD40 (25), OX-40 (32, 33), and CD27 (34). Thus, TRAF2 may inhibit TRAF3 or other molecule(s) from exerting a negative effect in synergy. Our results also underscore the importance of studying receptor-signal molecular interactions at physiological levels in relevant cell types.

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