Recruitment of CD40, TRAF2 and TRAF3 to membrane microdomains during CD40 signaling*

Bruce S. Hostager‡, Ian M. Catlett‡, and Gail A. Bishop‡§¶. Depts. of ‡Microbiology and §Internal Medicine, The University of Iowa, and ¶VA Medical Center, Iowa City, IA 52242

Running Title: Recruitment of CD40, TRAF2 and TRAF3 to membrane microdomains

Corresponding author: Gail A. Bishop, PhD.
3-570 BSB
University of Iowa
Iowa City, IA 52242
(319) 335-7945
Fax: (319) 335-9006
E-mail: gail-bishop@uiowa.edu
Summary

Signals delivered to antigen-presenting cells through CD40 are critical for the activation of immune responses. Intracellular TNF receptor-associated factors (TRAFs) are key elements of the signal transduction pathways of many TNF receptor family members, including CD40. We show for the first time that engagement of CD40 in intact B cells induces the rapid translocation of TRAF2 from the cytoplasm to the plasma membrane. We find that CD40 engagement also results in its recruitment, together with TRAF2 and TRAF3, to membrane microdomains, regions of the plasma membrane enriched in signaling molecules such as the Src family kinases. Using a membrane-permeable chelator of zinc or a mutant TRAF2 molecule we show that the putative zinc-binding domains of the TRAFs contribute to their recruitment to microdomains and to the downstream activation of c-Jun N-terminal kinase. We suggest that the zinc RING and zinc finger domains of the TRAFs are required for communication between CD40 and microdomain-associated signaling molecules, and may serve a similar role in the signal transduction pathways of other TNF receptor family members.
Introduction

Upon its ligation by CD154 on activated T lymphocytes, CD40 delivers essential activation signals to antigen presenting cells. In B lymphocytes, CD40 signals contribute to the activation of proliferation, differentiation, isotype switching and development of memory cells (1). In other types of antigen presenting cells, CD40 signals have recently been shown to participate in the activation of cell-mediated immune responses (2,3). CD40 ligation results in the stimulation of several important signaling molecules, including NF-κB (4), c-Jun N-terminal kinase (JNK) (5), and p38 (6). Members of the TNF receptor-associated factor (TRAF) family of proteins bind the cytoplasmic domain of CD40 and potentially initiate signal transduction (7-10). Understanding the roles of TRAF proteins in CD40 signaling requires careful characterization of the molecular interactions between CD40 and TRAF proteins. It has been suggested that TRAF2 is bound to CD40 in unstimulated B cells, and is released into the cytoplasm upon CD40 ligation (11). However, using similar techniques, others have concluded that the TRAFs are recruited to CD40 during signaling (12). To address the issue of subcellular localization in intact cells, we stably transfected a mouse B cell line with green fluorescent protein (GFP)-labeled TRAF2. Using confocal microscopy, we found that TRAF2 is distributed throughout the cytoplasm of unstimulated cells, and is recruited to the plasma membrane only following CD40 engagement with membrane-bound CD154 or anti-CD40 antibody.

Remarkably, we also find that CD154-stimulation of B cells renders CD40, TRAF2 and TRAF3 largely insoluble in non-ionic detergents. Recent reports have described the association of important signaling molecules in lymphocytes with detergent-insoluble, low density, membrane microdomains (13-16) or "rafts", enriched in sphingolipids, cholesterol, and glycosylphosphatidylinositol (GPI)-linked proteins (17). Using density gradient centrifugation, we demonstrate that CD40 engagement results in the rapid, dramatic recruitment of TRAF2 and TRAF3 to these specialized regions of the plasma membrane. In addition, we found that a mutant TRAF2 molecule containing a deletion in its zinc-binding domain displayed reduced raft recruitment, as did wild-type TRAF2 and TRAF3 in cells treated with a membrane-permeable chelator of zinc. Zinc chelation also blocked CD40-mediated JNK activation, but not the activation of JNK by osmotic stress. Taken together, these findings suggest that the zinc-binding domains of the TRAFs mediate interactions with raft components critical for downstream signaling events.
Experimental Procedures

DNA constructs- To construct hTRAF2 with an amino-terminal GFP tag, TRAF2 cDNA was ligated into the pEGFP-C2 vector (Clontech, Palo Alto, CA). The insert encoding TRAF2-GFP was subcloned into the inducible expression vector pOPRSVI.mcs1 (18), generating pTRAF2-GFP. Lac repressor (LacR) binding sites in the RSV promoter of this construct allow for the negative regulation of protein production by LacR. pEFLac encoding LacR has been described (19). TRAF2ΔZn (amino acids 199-501 of mouse TRAF2) was produced by PCR mutagenesis and placed into pOPRSVI.mcs1.

Cells- The mouse B cell lines M12.4.1 and CH12.LX, and transfectants expressing various hCD40 molecules have been described (20), as have transfectants expressing LacR (19). M12.LacR was stably transfected with pTRAF2-GFP to generate M12.TRAF2-GFP. CHO-K1 cells were from The American Type Culture Collection (ATCC, Manassas, VA). The CHO-mCD154 cell line was described previously (18). CHO cells expressing human CD154 (CHO-hCD154) were provided by Dr. Amelia Black (IDEC Pharmaceuticals Corp., San Diego, CA).

Transfections- Stable transfections of mouse B cell lines were carried out using electroporation as previously described (21).

Reagents- Nycodenz was from Pharmacia (Piscataway, NJ). Isopropylthio-β-D-galactoside (IPTG) was from Gibco BRL (Gaithersburg, MD). Tetrakis-(2-pyridylmethyl) ethylenediamine (TPEN) and 1,2-bis(o-Aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid tetra(acetoxyethyl) ester (BAPTA/AM) were purchased from Calbiochem (La Jolla, CA), and dissolved in DMSO. Brij-58 was from Pierce Chemical (Rockford, IL) and NP-40 from Polysciences, Inc. (Warring, PA). Horse radish peroxidase (HRP), HRP-labeled cholera toxin B subunit, and methyl-β-cyclodextrin were from Sigma Chemical Co. (St. Louis, MO).

Antibodies- The 1C10 hybridoma (anti-mCD40, rat IgG2a)(22) was provided by Dr. Frances Lund, Trudeau Institute, Saranac Lake, NY. mAb EM-95.3 (anti-mouse IgE, rat IgG2a isotype control)(23) was a gift of Dr. Thomas Waldschmidt, University of Iowa. The G28-5 (anti-hCD40) hybridoma was from the ATCC. MOPC-21 (mouse IgG1 isotype control) was from Sigma. Polyclonal rabbit Abs to TRAF2, TRAF3, TRAF6 and Lyn were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). For staining hCD40 on Western blots, sheep polyclonal anti-GST-hCD40 (extracellular domain) was prepared by
Elmira Biologicals (Iowa City, IA). HRP-labeled secondary Abs were from Bio-Rad (Hercules, CA). Lissamine rhodamine (LR)-labeled goat anti-rat IgG was from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA).

Confocal Microscopy- M12.TRAF2-GFP cells were cultured for 48 hrs. with 100 µM IPTG to induce TRAF2-GFP. For staining surface CD40 on unstimulated M12.TRAF2-GFP cells, cells were fixed in 2% paraformaldehyde for 20 minutes on ice, washed, then incubated with anti-mCD40 (1C10) and LR-labeled secondary Ab. For staining CD40 on activated cells, live cells were incubated for 10 min. (at 25°C or 37°C) with 10 µg/ml anti-mCD40 and 1:250 LR-secondary Ab, then fixed with 2% paraformaldehyde. CHO-K1 and CHO-mCD154 cells were stained for 15 min. at 37°C with 330 ng/ml 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, Eugene, OR). B cells (1x10⁶) and DiI-stained CHO (2 x 10⁵) cells were mixed in a final volume of 100 µl and incubated for 10 min. at 37°C, then examined by confocal microscopy (Bio-Rad MRC-1024 confocal system on a Nikon Optiphot microscope, 60X oil-immersion objective lens).

Cell Fractionation and Western blotting- Cells (10⁷/condition/ml) were stimulated for 10 min. at 37°C with 10 µg/ml anti-CD40 (G28-5 or 1C10) or isotype control mAb (MOPC-21 or EM95), then pelleted by centrifugation. Cells were resuspended in 200 µl ice-cold lysis buffer (1% Brij 58, 20 mM Tris pH 7.5, 150 mM NaCl, with protease and phosphatase inhibitors) and incubated on ice for 30 min. Lysates were centrifuged at 4°C and 14,000 x g for 25 min. After collecting supernatants, the detergent-insoluble pellets were resuspended and briefly sonicated in 200 µl lysis buffer supplemented with 0.5% SDS and 1% β-mercaptoethanol. In some experiments, cells were preincubated in serum-free medium (2 x 10⁶/ml) with 10 µM TPEN, 10 µM BAPTA/AM, or 1:2000 DMSO for 30 min. at 37°C. Cells were pelleted, resuspended to 10⁷/ml, and stimulated as above. Lysate fractions were diluted 1:2 with reducing SDS-PAGE loading buffer, heated for 5 min. at 95°C, subjected to SDS-PAGE, and electroblotted to PVDF membranes. To allow better detection of hCD40 on Western blots, lysate samples were deglycosylated prior to SDS-PAGE using PNGase F (New England Biolabs, Beverly, MA) according to the manufacturer’s protocol. A chemiluminescent substrate (Pierce Chemical Co., Rockford, IL) was used to detect HRP-labeled antibodies on Western blots.
Preparation of total cell extracts- M12.4.1 cells (10⁷/condition/ml) were stimulated for various periods of time at 37°C with 10 µg/ml anti-mCD40 mAb or isotype control mAb, then pelleted by centrifugation. Cells were resuspended in 200 µl 2X Laemmli sample buffer (1% SDS, 62.5 mM Tris pH 6.8, 15% glycerol, 0.2% β-mercaptoethanol), sonicated briefly (to reduce viscosity), heated (5 min. at 95°C) and subjected to SDS PAGE/Western blotting as above.

Raft isolation- Raft isolation was accomplished using a combination of published protocols (15,24). B cells (2.5 x 10⁷/condition/ml) were stimulated at 37°C for 10 min. using 10 µg/ml anti-CD40 mAb or 5 x 10⁶ CD154-expressing CHO cells. In some experiments, cells were pretreated with TPEN (as above) or with MCD. Pretreatment of B cells with MCD was essentially as described (14). Briefly, cells were washed in serum free medium, resuspended in serum-free medium containing 22 mg/ml MCD (approximately 20 mM), incubated 10 minutes at room temperature, 15 minutes at 37°C, then stimulated with anti-hCD40 antibody (as above). Following stimulation, cells (including CHO-hCD154 stimulators, where used) were pelleted and resuspended in 400 µl ice-cold lysis buffer and incubated on ice for 30 minutes. Cell lysates were diluted 1:2 with 70% Nycodenz in 20mM Tris, pH 7.5, 150 mM NaCl and transferred to 3 mL ultracentrifuge tubes. Lysates were overlayed with a Nycodenz step gradient (200 µl each of 25%, 21.5%, 18%, 15% and 8% Nycodenz) (24), and centrifuged for 4 hrs. at 200,000 x g at 4°C (Beckman SW60 rotor). Eleven 200 µl fractions were removed from the tubes, starting at the top (lowest density), then diluted 1:2 in reducing SDS-PAGE loading buffer and heated for 5 minutes at 95°C.

Samples of fractions were subjected to SDS-PAGE, then electroblotted to PVDF membranes. For testing ganglioside GM1 content of density gradient fractions, 2.5 x 10⁷ unstimulated M12.4.1 cells were incubated for 10 minutes at room temperature in 1 ml of culture medium containing 3.4 µg cholera toxin B subunit-HRP (or, as controls, untreated cells and cells incubated with an equivalent amount of unconjugated HRP). Cells were then subjected to lysis and density gradient centrifugation as above. Gradient fractions were assayed for peroxidase activity by mixing 10 µl gradient fraction with 100 µl 50 mM sodium phosphate, 25 mM citric acid, pH 5.0, 1 mg/ml o-phenylenediamine dihydrochloride, 0.012% H₂O₂. Samples were incubated for approximately 5 minutes at room temperature and the reaction stopped.
by adding 150 μl 0.67 M sulfuric acid. Optical density of the samples was read at 405 nm in an ELISA plate reader.

**JNK activity assay**—M12-hCD40 cells (2 x 10⁶/ml/condition) were stimulated for 5 minutes at 37°C with 5 μg/ml anti-hCD40 mAb, isotype control mAb, or 0.6M sorbitol. Cell lysates were assayed for JNK activity as previously described (19). In some experiments, cells were incubated (10 minutes at room temperature, then 10 minutes at 37°C) in serum-free medium containing 27.5 mg/ml (approximately 25 mM) MCD before stimulation.
Results

Subcellular Localization of TRAF2 During CD40 Signaling- To examine the localization of TRAF2 in intact B cells, we stably transfected M12.LacR cells with a construct encoding an inducibly-expressed green fluorescent protein (GFP) tagged TRAF2 molecule, or as a control, inducible GFP. Confocal microscopy revealed that GFP alone was evenly distributed in cells, including the nucleus (Fig. 1A). Incubation of cells with anti-mouse CD40 (mCD40) and a rhodamine-labeled secondary Ab (10 min., 25°C, Fig. 1A-C) did not affect the distribution of GFP (unstimulated cells not shown). In contrast, TRAF2-GFP was excluded from the nuclei and localized to the cytoplasm of unstimulated cells (fixed, then surface stained with anti-mCD40 followed by a rhodamine-labeled secondary antibody, Fig. 1D-F). In cells stimulated for 10 min. at 25°C with anti-CD40 and rhodamine-labeled secondary Abs, TRAF2-GFP was localized to small patches in the plasma membrane (Fig. 1G-I), and colocalized with CD40 (colocalization in yellow, Fig. 1I). Recruitment of TRAF2-GFP from the cytoplasm to the plasma membrane began immediately upon the addition of anti-CD40 mAb, and was essentially complete after 5-10 minutes. Interestingly, Ab stimulation of cells at 37°C resulted in significant internalization of CD40 with associated TRAF2 (Fig. 1J-L). However, internalization appeared minimal when B cells were stimulated at 37°C for 10 minutes with membrane-bound CD154 (Fig. 1M-O). We conclude that in intact B cells, TRAF2 associates with CD40 only following CD40 ligation.

Association of TRAF2 and CD40 with Detergent-Insoluble Complexes- Although confocal microscopy indicated that CD40 and TRAF2 associate in activated cells, we were unable to consistently demonstrate this association using conventional co-immunoprecipitation techniques. Previously however, it was reported that the total amount of TRAF2 that could be immunoprecipitated from stimulated cells was considerably less than that obtained from unstimulated cells (12). It was suggested that stimulation either resulted in the recruitment of the TRAFs to detergent-insoluble complexes or that the TRAFs undergo activation-induced degradation. To determine if TRAF2 becomes associated with detergent-insoluble material in activated cells, we examined the effects of CD40 stimulation on a mouse B cell line transfected with human CD40 (hCD40), M12.hCD40 (20). Cells were stimulated with anti-hCD40 mAb, lysed in 1% Brij 58 (a relatively mild, non-ionic detergent), and lysates centrifuged to pellet detergent-insoluble
material. The insoluble material was dissolved in 0.5% SDS/1% β-mercaptoethanol so that it could be subjected to SDS PAGE/Western blot analysis and compared to samples of the Brij 58-soluble material. On Western blots, the majority of TRAF2 from unstimulated B cells appeared in the detergent-soluble fraction (Fig. 2A). However, in cells stimulated through hCD40 or endogenous mouse CD40 (mCD40), much of the TRAF2 became associated with the detergent-insoluble fraction (Fig. 2A). Interestingly, a portion of the TRAF2 in the insoluble fraction appeared to be post-translationally modified, resulting in the appearance of multiple high molecular weight forms. To determine if CD40 itself becomes detergent-insoluble in stimulated cells, we generated a polyclonal antiserum for detecting hCD40 on Western blots. In unstimulated cells, a significant fraction of CD40 was already present in the detergent-insoluble fraction (Fig. 2). Upon stimulation, additional CD40 was recruited to this fraction (Fig. 2A), although this recruitment was not as dramatic as the activation-induced partitioning of TRAF2 (Fig. 2A). Association of hCD40 (or TRAF2) with the detergent-insoluble fraction did not occur if the stimulating Ab was instead added post-lysis (data not shown). To determine if the recruitment of CD40 to detergent-insoluble material is dependent upon TRAF association, we repeated the experiment using M12.4.1 cells expressing a mutant hCD40 molecule with a 55 amino acid cytoplasmic deletion (hCD40Δ55) (20). hCD40Δ55 lacks the binding sites for TRAFs 2, 3, and 6 (10,25,26), and has no signaling activity (20). As with full-length CD40, engagement of hCD40Δ55 resulted in its recruitment to detergent-insoluble material, indicating that this activation-dependent event does not require TRAF binding (Fig. 2B). While engagement of hCD40Δ55 did not mediate recruitment of TRAF2 to the insoluble fraction, engagement of endogenous mCD40 in the same cells induced the transition (Fig. 2B).

To test the possibility that TRAF2 may also undergo degradation in activated cells, we stimulated M12.4.1 cells for various periods of time with anti-mCD40 mAb, then we prepared cell lysates using either 1% Brij 58 lysis buffer, or buffer containing 1% SDS to more completely solubilize cellular proteins. With a stimulation period of as little as one minute, most of the TRAF2 appears in the Brij 58-insoluble fraction (Fig 2C). However, total cellular TRAF2 amounts also decrease with stimulation (Fig 2D). We conclude that TRAF2 is both recruited to Brij 58-insoluble material and undergoes degradation as a result of CD40 stimulation.
Recruitment of CD40, TRAF2 and TRAF3 to Membrane Rafts - T cell receptor (TCR) ligation induces recruitment of Zap-70, TCRα, CD3ε and CD3ζ to membrane rafts, which may facilitate their interactions with raft-associated Src-family kinases (14,15). Potentially, the stimulation-induced insolubility of CD40 and TRAF2 results from a similar association with low-density, detergent-insoluble lipid rafts. To test this hypothesis, we stimulated mouse B cells for 10 min. at 37°C with hCD154-bearing CHO cells, lysed the cells in 1% Brij 58, and fractionated the lysates by density gradient ultracentrifugation. Insoluble membrane rafts are found in the low density portion of the gradient, while soluble membrane and cytoplasmic proteins are found in higher density fractions (15). TRAF2 from unstimulated M12.hCD40 cells was found in the high density fractions (Fig. 3A). CD40 engagement resulted in a marked shift of TRAF2 into lower density fractions, suggesting an association with membrane rafts. Similar results were obtained using anti-mCD40, anti-hCD40 or CHO-mCD154 cells as stimuli, and in a second mouse B cell line CH12.LX (not shown). Preliminary experiments indicate that this recruitment also occurs in normal mouse splenic B cells. Blots were also examined for two additional CD40-binding proteins, TRAF3 and TRAF6, both of which appeared in the high density fractions from unstimulated cells (Fig. 3B and C). In CD154-stimulated cells, the behavior of TRAF3 was similar to that of TRAF2, while there was no detectable association of TRAF6 with membrane rafts. Stimulation of M12.hCD40 cells with hCD154 also induced recruitment of hCD40 to low density fractions, although a small amount of CD40 was found in the low density fractions of unstimulated cells as well (Fig. 3D). In addition, we confirmed that TRAF2-GFP was recruited to low-density membrane rafts in CD40-stimulated cells (not shown), and that the raft recruitment of TRAF2 also occurred at 25°C (Fig. 3E, F). To examine the localization of a known constituent of membrane rafts, Western blots were reprobed for Lyn (Fig. 3G). As expected based upon previous reports (27), Lyn localized to low density fractions. Lyn distribution was similar in unstimulated cells (not shown). The ganglioside GM1 has also been reported to be enriched in membrane microdomains (28). To test density fractions for GM1 content, cells were incubated with cholera toxin (which binds GM1) conjugated to HRP, washed, then subjected to lysis and density gradient centrifugation. As shown in Fig 3H, peroxidase activity was detected in all fractions, but was considerably enriched in the low-density material. Taken together, these results support the hypothesis that CD40, TRAF2 and TRAF3 are recruited to membrane rafts in activated B lymphocytes.
**Effects of methyl-β-cyclodextrin on CD40 signaling.** It has been reported that cholesterol-binding agents, such as methyl-β-cyclodextrin (MCD), can disrupt membrane microdomains (29). Treatment of live cells with MCD disrupts T cell receptor-mediated Ca ++ fluxes, and it has been argued that this type of experiment demonstrates the importance of membrane microdomains in TCR signaling (14,30). However, we found that MCD treatment, while rendering more than 10% of B cells permeable to trypan blue (not shown), only partially disrupted membrane rafts. As shown in Fig 4A, MCD treatment of M12.hCD40 cells dissociated only a portion of Lyn from low-density Brij 58-insoluble complexes. A previous report similarly demonstrated that MCD was not able to entirely dissociate Fyn (another Src-family kinase) from detergent-insoluble complexes, and it was suggested that MCD may only inefficiently extract cholesterol from the inner leaflet of the plasma membrane (29). We also found that the raft association of TRAF2 in CD40-stimulated cells (Fig 4B) was only partially disrupted by MCD. However, the combination of MCD treatment and lysis in a stronger detergent solution (1% Triton X-100) did lead to more complete dissociation of membrane microdomains (Fig 4C). We next examined the effects of MCD on the activation of JNK, an enzyme rapidly activated by CD40 stimulation. In addition to its inability to fully disrupt membrane microdomains (unless combined with a sufficiently strong detergent), MCD treatment, by itself, activated JNK (Fig. 4D). MCD treatment appeared not to specifically inhibit JNK activation mediated by CD40 (Fig. 4D), but it is difficult to know if there was a partial inhibition masked by the MCD-induced activation of JNK, or if MCD was unable to sufficiently disrupt raft complexes involved in CD40 signaling. Taken together, our results indicate it would be inappropriate to draw firm conclusions from CD40-signaling experiments performed using MCD-treated cells.

**Zinc-dependent JNK Activation and Recruitment of TRAFs to Rafts.** In addition to its carboxy-terminal receptor (CD40)-binding domain, TRAF2 contains amino-terminal zinc (Zn) RING and Zn finger motifs (31). Although the functions of these putative Zn-binding domains are unclear, similar domains in other proteins can mediate protein-protein interactions (32). Mice transgenic for a mutant TRAF2 molecule lacking the Zn RING domain (33) display defective activation of JNK by TNFR family members, including CD40. These results, and mutational analysis of TRAF2 (34), strongly suggest that the Zn-binding features...
of the TRAFs are important for interactions with downstream signaling molecules. In support of this hypothesis, we found that tetrakis-(2-pyridylmethyl) ethylenediamine (TPEN), a membrane-permeable chelator of Zn (35) abrogated the CD40-mediated activation of JNK (Fig. 5A), but did not inhibit the activation of JNK by osmotic stress (sorbitol). A cell-permeable chelator of Ca++, BAPTA/AM, did not specifically inhibit the activation of JNK by CD40, indicating that the action of TPEN is not due to its low affinity for Ca++. Although TPEN may affect other steps in the CD40 signal transduction pathway, its primary effects appear to be on TRAF function. Zn chelation abolished the activation-induced post-translational modification of TRAF2 and significantly inhibited the recruitment of TRAF2 (Fig. 5B), but not CD40 (not shown), to raft complexes. Similarly, a mutant TRAF2 molecule in which we deleted most of the Zn-binding domain (TRAF2ΔZn) displayed reduced recruitment to membrane rafts (Fig. 5C).

Importantly, TPEN treatment did not further reduce recruitment of TRAF2ΔZn to membrane rafts, indicating that Zn chelation does not interfere with binding of the TRAF domain of TRAF2 to the cytoplasmic domain of CD40. Together, these results suggest the Zn-binding features of TRAF2 interact with membrane raft-associated molecules, and that these interactions are required for downstream signaling events. Further support for this hypothesis is presented in Figure 6. Cells were stimulated with anti-CD40 then solubilized in 1% Brij 58 or lysis buffer in which Brij 58 was replaced with 0.5% NP-40. Although NP-40 was able to entirely solubilize CD40, a significant proportion of the TRAF2 and TRAF3 from stimulated cells remained in the detergent insoluble material, indicating that the TRAFs may bind an NP-40-insoluble microdomain component. Pretreatment of cells with TPEN abolished CD40-stimulated recruitment of the TRAFs to the NP-40-insoluble fraction, demonstrating that the Zn-binding domains of the TRAFs are critical for their association with the insoluble fraction.
Discussion

We show that engagement of CD40 results in the rapid recruitment of TRAF2 and TRAF3 from the cytosol to the plasma membrane where they associate with membrane rafts. CD40 stimulation also induces the association of CD40 itself with membrane rafts, but its recruitment does not appear TRAF-dependent. TRAF6 has also been characterized as a CD40-binding protein, yet in contrast to TRAFs 2 and 3, showed little if any raft recruitment during stimulation of B cells. However, it remains possible that TRAF6 is recruited only after prolonged stimulation, or at low but functionally significant levels.

Although the functions of the TRAF proteins remain enigmatic, they appear to serve as adapter molecules linking CD40 to important components of its signaling pathway. Previous work indicates that the Zn RING and Zn fingers of TRAF2 are not required for its binding to CD40 (7,26). However, we found that disrupting the function of the Zn-binding domains (with TPEN or by mutation) partially inhibited the raft recruitment of TRAF2 and TRAF3, suggesting that raft-associated molecules (in addition to CD40) contribute to the avidity of TRAF-microdomain interactions. This hypothesis is supported by the fact that the CD40 from activated cells was completely solubilized in 0.5% NP-40, while the same detergent only partially solubilized TRAFs 2 and 3.

While our results indicate that TRAFs 2 and 3 may associate with membrane raft constituents, these molecules remain to be identified. Recently, putative TRAF-interacting proteins have been identified, including apoptosis signal-regulating kinase (ASK1) (36), NF-κB-inducing kinase (NIK) (37), germinal center kinase (GCK)(38), and MEKK1, which, unlike the other kinases, may interact with the Zn-binding features of TRAF2 (39). However, the interactions of these kinases with TRAFs have been almost exclusively demonstrated in epithelial cell lines overexpressing transiently-transfected TRAFs and candidate molecules. Using commercial antisera, we have been unable to detect the endogenous forms of these putative TRAF-interacting molecules in detergent-insoluble TRAF-containing fractions from CD40-stimulated B cells. Further work is necessary to determine if there are very low levels of these kinases present in the CD40 signal transduction complex or if other proteins (perhaps related to the candidates previously identified) mediate CD40 signaling in B cells. Interestingly, one raft-associated kinase, Lyn, has been postulated to participate in CD40 signaling (40,41), although there is currently no evidence that it directly interacts with any of the TRAF molecules.
One key to identifying TRAF-interacting molecules in membrane microdomains may be the post-translational modification displayed by raft-associated TRAFs (Figs. 2-6). The modification appears to require the Zn-binding features of the TRAFs, in that it is largely ablated by TPEN treatment and is not evident in TRAF2ΔZn. If this modification is important for the binding of raft-associated molecules, such molecules may have been overlooked in previous attempts to identify TRAF binding partners.

Furthermore, the modification may require the activity of raft-associated proteins, and determining the type of modification should facilitate their identification. Interestingly, several Zn RING-containing proteins, such as Cbl, participate in the ubiquitination of themselves and other proteins, targeting them for degradation (42). In light of the activation-induced modification and degradation of TRAF2 we observe, it seems reasonable to suggest that one or more of the Zn RING-containing TRAFs similarly promote ubiquitination, and perhaps help to limit the duration of CD40 signaling. We are currently testing this hypothesis.

The discovery of raft-associated CD40, TRAF2 and TRAF3 represents a major advance in the understanding of CD40 signal transduction, indicating where and how to look for other molecules that mediate the proximal steps in CD40 signaling. While our work focuses on the CD40 signal transduction pathway, the results presented here have broad implications for signaling through a variety of related receptors. TRAF molecules interact with several TNF receptor family members, including TNF-R1, TNF-R2, CD27, CD30 and RANK, (31,43-46) and in each case may mediate important interactions with microdomain-associated signaling molecules.

Acknowledgements

We thank Dr. Gary Koretzky and Dr. Robert Deschenes for critical review of the manuscript, and Thomas Moninger for valuable assistance with confocal microscopy.
References


Footnotes

*This work was supported by an Arthritis Foundation Postdoctoral Fellowship awarded to B.S.H., grants to G.A.B. from the NIH (AI28847, CA66570), and the Veterans Administration (Merit Review 383). Core support was provided by NIH grant DK25295 to the U. of Iowa Diabetes and Endocrinology Research Center.

1 Abbreviations used in this paper: ASK1, apoptosis signal-regulating kinase; BAPTA/AM, 1,2-bis(o-Aminophenoxy)ethane-N,N',N'-tetraacetic acid tetra(acetoxyethyl) ester; CT-HRP, cholera toxin B subunit-horse radish peroxidase conjugate; GCK, germinal center kinase; GFP, green fluorescent protein; IPTG, isopropylthio-β-D-galactoside; JNK, c-Jun N-terminal kinase; mAb, monoclonal antibody; MCD, methyl-β-cyclodextrin; TPEN, tetrakis-(2-pyridyldimethyl) ethylenediamine; TRAF, TNF receptor-associated factor.
Figure Legends

FIG. 1. CD40 engagement induces CD40, TRAF2 colocalization. Confocal microscopy was used to visualize an optical section through the midpoint of a M12.4.1 B cell transfected with GFP alone (M12.GFP cells) stimulated for 10 minutes at room temperature with anti-mouse CD40 and a lissamine-rhodamine (LR)-labeled secondary Ab (A, GFP; B, CD40 staining; C, overlay). Scale bar in A represents 10 μm. In panels D-F, M12.4.1 cells stably transfected with hTRAF2-GFP (M12.TRAF2-GFP cells) were paraformaldehyde fixed, then stained with anti-mCD40 and LR-labeled secondary Ab (D, TRAF2-GFP; E, CD40 staining; F, overlay). Panels G-I show M12.TRAF2-GFP cells stimulated for 10 minutes at room temperature with anti-mCD40 and LR-labeled secondary Ab, then paraformaldehyde fixed (G, TRAF2-GFP; H, CD40 staining; I, overlay). In J-L, M12.TRAF2-GFP cells were incubated for 10 minutes at 37°C with anti-mCD40 and LR-labeled secondary Ab, then fixed (J, TRAF2-GFP; K, CD40 staining; L, overlay). Panels M-O show M12.TRAF2-GFP cells incubated for 10 minutes at 37°C with DiI-labeled CHO-mCD154 cells (M, TRAF2-GFP; N, DiI staining; O, overlay). In this and all subsequent figures, experiments were performed at least twice with similar results.

FIG. 2. CD40 engagement induces association of TRAF2 and CD40 with detergent-insoluble complexes, and degradation of TRAF2. Detergent (Brij 58)-soluble and detergent-insoluble fractions from M12.hCD40 cells stimulated with isotype control mAb (i), anti-hCD40 (h) or anti-mCD40 (m) were prepared, resolved by SDS PAGE and transferred to PVDF membranes. Membranes were Western blotted for TRAF2 (A, upper panel) and hCD40 (A, lower panel). A similar experiment was performed with M12.hCD40Δ55 cells (B). In A and B, CD40 was deglycosylated prior to SDS PAGE to improve its detection on Western blots. In C, M12.4.1 cells were stimulated for various periods of time with anti-mCD40 mAb, Brij-soluble and insoluble fractions prepared and Western blotted for TRAF2, as in A. For the zero time point, cells were treated for 1 minute with isotype control mAb prior to lysis. Longer incubations with isotype control mAb gave equivalent results (not shown). To examine activation-induced degradation of TRAF2, M12.4.1 cells were stimulated for various periods of time with anti-mCD40 mAb and total cell extracts prepared using lysis buffer containing SDS and β-mercaptoethanol. Total cell extracts were resolved by SDS PAGE, transferred to PVDF membranes and Western blotted for TRAF2.
Cells for the zero time point were treated for 2 minutes with isotype control mAb prior to lysis. TRAF2 Western blots were stripped and reblotted for actin to show equal lane loading (D, lower panel).

**FIG. 3.** CD40 engagement induces the association of CD40, TRAF2 and TRAF3 with low-density complexes. M12.hCD40 cells were stimulated for 10 minutes at 37°C with CHO-hCD154 (or untransfected CHO cells as the negative control), then lysed in 1% Brij 58 and subjected to density gradient centrifugation. Western blots of density gradient fractions were evaluated for TRAF2 (A). Fraction density increases from left to right. Blots were reprobed for (B) TRAF3, and (C) TRAF6. Density gradient fractions from the same experiment were deglycosylated, run on separate SDS PAGE gels and Western blotted for hCD40 (D). Anti-hCD40 (αhCD40) stimulation of M12.hCD40 cells at either 37°C (E), or 25°C (F) also induced recruitment of TRAF2 to low density fractions. Gradient fractions were also examined for known constituents of membrane microdomains, Lyn (G) and GM1 (H). Panel G is a Lyn Western blot of gradient fractions from αhCD40-stimulated M12.hCD40 cells (Lyn distribution was similar in unstimulated cells, not shown). GM1 content of density gradient fractions (unstimulated M12.4.1 cells), was measured by bound CT-HRP activity (H), as described in Experimental Procedures.

**FIG. 4.** Affects of MCD treatment on membrane raft buoyancy and CD40-stimulated JNK activity. M12.hCD40 cells were preincubated with (+) or without (-) MCD, stimulated for 10 minutes at 37°C with anti-hCD40 mAb, then lysates prepared using 1% Brij 58. Lysates were subjected to density gradient fractionation, SDS PAGE and Western blotting for Lyn (A). Western blot membranes from A were reprobed for TRAF2 (B). In panel C, showing a Western blot for Lyn, cells were treated as in A, except 1% Triton X-100 was substituted for Brij 58 in the lysis buffer. To examine the effects of MCD on the activation of JNK, M12.hCD40 cells incubated with or without MCD, stimulated for 5 minutes at 37°C with anti-hCD40 mAb (+), an isotype control mAb (-), or 0.6 M sorbitol (S). Cell lysates were then tested for activated JNK using GST-cJun (1-79) as a substrate. Phosphorylated GST-Jun appears as a band on the autoradiogram shown in D.
FIG. 5. Contributions of zinc to CD40-mediated activation. To determine if chelation of Zn\(^{++}\) inhibits CD40-induced JNK activation, M12.hCD40 cells were preincubated with 10 \(\mu\)M TPEN, 10 \(\mu\)M BAPTA/AM or 1:2000 DMSO, then stimulated for 5 minutes at 37°C with 5 \(\mu\)g/ml isotype control mAb (-), anti-hCD40 (+), or 0.6 M sorbitol (S). Lysates were tested for activated JNK using GST-cJun (1-79) as a substrate. Phosphorylated GST-Jun appears as a band on the autoradiogram shown in A. To determine if TPEN also inhibits the CD40-mediated raft recruitment of TRAF2, M12.hCD40 cells were pretreated with TPEN or DMSO then stimulated with isotype control mAb or anti-hCD40 mAb. Cell lysates (1% Brij) were subjected to density gradient centrifugation, and Western blot analysis for TRAF2 (arrow) (B, density of fractions increases from left to right). To confirm that the zinc-binding features of TRAF2 contribute to raft recruitment, M12.4.1 cells transfected with inducible-TRAF2\(\Delta\)Zn were cultured for 48 hrs. with 100 \(\mu\)M IPTG to upregulate production of the TRAF2 mutant, incubated for 30 minutes with TPEN or DMSO, then stimulated for 10 min. (37°C) with isotype control mAb (-) or anti-mCD40 (+). Detergent-soluble and insoluble fractions were examined for TRAF2 content on Western blots (C).

FIG. 6. Dissociation of CD40 but not TRAFs from membrane microdomains by 0.5% NP-40.

M12.hCD40 cells were stimulated with anti-hCD40 (+) or an isotype control mAb (-), then lysed in buffer containing either 1% Brij 58 or 0.5% NP-40. Detergent soluble and insoluble fractions were isolated and subjected to SDS-PAGE and Western blot analysis for TRAF2, TRAF3 and hCD40.
Fig. 2
Hostager, B.S.

A


Min. of stimulation

B

C

D

TRAF2
hCD40
TRAF2
hCD40Δ55
TRAF2
(soluble)
TRAF2
(insoluble)

Min. of stimulation

actin
Fig. 4
Hostager, B.S.
Fig. 3
Hostager, B.S.
Fig. 6
Hostager, B.S.

<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>TPEN</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anti-hCD40:</strong></td>
<td>Sol.</td>
<td>Ins.</td>
</tr>
<tr>
<td><strong>1% Brij 58</strong></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>TRAF2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRAF3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD40</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>TPEN</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anti-hCD40:</strong></td>
<td>Sol.</td>
<td>Ins.</td>
</tr>
<tr>
<td><strong>0.5% NP-40</strong></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>TRAF2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRAF3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD40</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 5
Hostager, B.S.
Recruitment of CD40 TRAF2 and TRAF3 to membrane microdomains during CD40 signaling
Bruce S. Hostager, Ian M. Catlett and Gail A. Bishop

J. Biol. Chem. published online March 15, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M909520199

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 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2000/03/15/jbc.M909520199.citation.full.html#ref-list-1