Recruitment of CD40 and Tumor Necrosis Factor Receptor-associated Factors 2 and 3 to Membrane Microdomains during CD40 Signaling*

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Signals delivered to antigen-presenting cells through CD40 are critical for the activation of immune responses. Intracellular tumor necrosis factor (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 found 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 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 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.

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. In other types of antigen-presenting cells, CD40 signals have been shown to participate in the activation of cell-mediated immune responses. CD40 ligation results in the stimulation of several important signaling molecules, including nuclear factor κB (4), c-Jun N-terminal kinase (JNK) (5), and p38 (6). Members of the tumor necrosis factor (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 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 found 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 low density, detergent-insoluble membrane microdomains (13–16) or “rafts,” enriched in sphingolipids, cholesterol, and glycosphingolipid/lipid-protein complexes (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 TRAFs mediate interactions with raft components critical for downstream signaling events.

EXPERIMENTAL PROCEDURES

DNA Constructs—To construct human TRAF2 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. LacR-binding sites in the Rous sarcoma virus 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 polymerase chain reaction mutagenesis and placed into pOPRSVI.mcs1.

body: GST, glutathione S-transferase; Dii, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; PAGE, polyacrylamide gel electrophoresis; MEKK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase.
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 LacZ (19). M12.LacR was stably transfected with pTRAF2-GFP to generate M12.TRAF2-GFP. CHO-K1 cells were from American Type Culture Collection (Manassas, VA). The CHO-mCD154 cell line was described previously (18). Immunoprecipitating human CD154 (CHO-hCD154) were provided by Dr. Amelia Black (IDE C Pharmaceuticals Corp., San Diego, CA).

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

Reagents—Nycozen was from Amersham Pharmacia Biotech. Isopropylb-thiogalactopyranoside was from Life Technologies, Inc. N,N,N’,N’-Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) and 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid (BAPTA-AM) were purchased from Calbiochem and dissolved in MeSO. Brij 58 was from Pierce, and Nonidet P-40 was from Polysciences, Inc. (Warrington, PA). Horseradish peroxidase (HRP), HRP-labeled cholera toxin B subunit, and methyl-b-cyclodextrin (MCD) were from Sigma.

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 American Type Culture Collection. MOPC-21 (anti-mCD154 isotype control) was from Sigma. Polyclonal rabbit antibodies 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) antibody was prepared by Elmira Biologicals (Iowa City, IA). HRP-labeled secondary antibodies were from Bio-Rad. Lissamine rhodamine-labeled goat anti-rat IgG was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Confocal Microscopy—M12.TRAF2-GFP cells were cultured for 48 h with 100 µM isoprpyl-b-thiogalactopyranoside to induce TRAF2-GFP. For staining surface CD40 on unstimulated M12.TRAF2-GFP cells, cells were fixed in 2% paraformaldehyde for 20 min on ice, washed, and then incubated with anti-mCD40 antibody (1C10) and lissamine rhodamine-labeled secondary antibody. For staining CD40 on activated cells, live cells were incubated for 10 min (at 25 or 37 °C) with 10 µg/ml anti-mCD40 antibody and 1:250 lissamine rhodamine-labeled secondary antibody and 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’-diocadecyl-3,3,3’,3’-tetramethyldiiodocyanobenzene chlorophorlate (DiI; Molecular Probes, Inc., Eugene, OR). B cells (1 × 106) and DiI-labeled CHO cells (2 × 106) were mixed (on a 1:1000 ratio) on a final volume of 100 µl, incubated for 10 min at 37 °C, and then examined by confocal microscopy.

Cell Fractionation and Western Blotting—Cells (106/ml/condition) were stimulated for 10 min at 37 °C with 10 µg/ml anti-CD40 antibody (G28-5 or 1C10) or isotype control mAb (MOPC-21 or EM-95) and then pelleted. Cells were resuspended in 200 µl of ice-cold lysis buffer (1% Brij 58, 20 mM Tris (pH 7.5), and 150 mM NaCl with protease and phosphatase inhibitors) and incubated on ice for 30 min. Lysates were centrifuged at 14,000 × g for 25 min at 4 °C. After collecting supernatants, the detergent-insoluble pellets were resuspended and briefly sonicated in 200 µl of lysis buffer supplemented with 0.5% SDS and 1% β-mercaptoethanol. In some experiments, cells were preincubated in serum-free medium (2 × 106/ml) with 10 µM TPEN, 10 µM BAPTA/AM, or 1:2000 MeSO for 30 min at 37 °C. Cells were pelleted, resuspended to 106/ml, and stimulated as described 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 onto polyvinylidene difluoride membranes. For testing ganglioside GM1 content of density gradient fractions, 2.5 × 107 unstimulated M12.4.1 cells were incubated for 10 min at room temperature in 1 ml of culture medium containing 3.4 µg of HRP-labeled cholera toxin B subunit (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 described above. Gradient fractions were assayed for peroxidase activity by mixing 10-µl fractions with 100 µl of 50 mM sodium phosphate, 25 mM citric acid (pH 5.0), 1 mg/ml o-phenylenediamine dihydrochloride, and 0.012% H2O2. Samples were incubated for 5 min at room temperature, and the reaction was stopped by adding 150 µl of 0.67 M sulfuric acid. Optical density of the samples was read at 405 nm in an enzyme-linked immunosorbent assay plate reader.

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 irudibly expressed 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-mCD40 antibody and a rhodamine-labeled secondary antibody (10 min at 25 °C) (Fig. 1, A–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 and then surface-stained with anti-mCD40 antibody followed by a rhodamine-labeled secondary antibody) (Fig. 1, D–F). In cells stimulated for 10 min at 25 °C with anti-CD40 and rhodamine-labeled secondary antibodies, TRAF2-GFP was translocated to small patches at the plasma membrane (Fig. 1, G–I) and colocalized with CD40 (colocalization in yellow) (Fig. 1J). 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 min. Interestingly, antibody stimulation of cells at 37 °C resulted in significant internalization of CD40 with associated TRAF2 (Fig. 1, J–L). However, internalization appeared minimal when B cells were stimulated at 37 °C for 10 min with membrane-bound CD154 (Fig. 1, M–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 coimmunoprecipitation techniques. Previously, however, it
CD40/TRAFF2/TRAFF3 Recruitment to Membrane Microdomains

FIG. 1. CD40 engagement induces CD40 and TRAF2 colocalization. Confocal microscopy was used to visualize an optical section through the midpoint of an M12.4.1 B cell transfected with GFP alone (M12.GFP cells) and stimulated for 10 min at room temperature with anti-mCD40 antibody and a lissamine rhodamine-labeled secondary antibody (A, GFP; B, CD40 staining; C, overlay). Scale bar = 10 μm. In D–F, M12.4.1 cells stably transfected with human TRAF2-GFP (M12.TRAF2-GFP cells) were paraformaldehyde-fixed and then stained with anti-mCD40 antibody and a lissamine rhodamine-labeled secondary antibody (D, TRAF2-GFP; E, CD40 staining; F, overlay). G–I show M12.TRAF2-GFP cells stimulated for 10 min at room temperature with anti-mCD40 antibody and a lissamine rhodamine-labeled secondary antibody and then paraformaldehyde-fixed (G, TRAF2-GFP; H, CD40 staining; I, overlay). In J–L, M12.TRAF2-GFP cells were incubated for 10 min at 37 °C with anti-mCD40 antibody and a lissamine rhodamine-labeled secondary antibody and then fixed (J, TRAF2-GFP; K, CD40 staining; L, overlay). M–O show M12.TRAF2-GFP cells incubated for 10 min 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.

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 resulted in the recruitment of TRAFs to detergent-insoluble complexes or that 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 hCD40, M12.hCD40 (20). Cells were stimulated with anti-hCD40 mAb and lysed in 1% Brij 58 (a relatively mild, non-ionic detergent), and lysates

FIG. 2. CD40 engagement induces the association of TRAF2 and CD40 with detergent-insoluble complexes and degradation of TRAF2. Detergent (Brij 58)-soluble (Sol.) and detergent-insoluble (Ins.) fractions from M12.hCD40 cells stimulated with isotype control mAb (i), anti-hCD40 antibody (h), or anti-mCD40 (m) antibody were prepared, resolved by SDS-PAGE, and transferred to polyanlinidene difluoride membranes. Membranes were Western-blotted for TRAF2 (A, upper panel) and hCD40 (lower panel). A similar experiment was performed with M12.hCD40×55 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, and Brij-soluble and -insoluble fractions were prepared and Western-blotted for TRAF2 as described for A. For the zero time point, cells were treated for 1 min 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 were prepared using lysis buffer containing SDS and mercaptoethanol. Total cell extracts were resolved by SDS-PAGE, transferred to polyanlinidene difluoride membranes, and Western-blotted for TRAF2 (D, upper panel). Cells for the zero time point were treated for 2 min with isotype control mAb prior to lysis. TRAF2 Western blots were stripped and rebotted for actin to show equal lane loading (D, lower panel).
ment 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 TRAF2, TRAF3, and TRAF6 (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). Although 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, and 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 1 min, most of the TRAF2 appeared in the Brij 58-insoluble fraction (Fig. 2C). However, total cellular TRAF2 amounts also decreased with stimulation (Fig. 2D). We conclude that TRAF2 is 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 ligation induces recruitment of Zap-70, T cell receptor ligation induces recruitment of Zap-70, T

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not further reduce recruitment of TRAF2ΔZn to membrane rafts, indicating that zinc chelation does not interfere with the binding of the TRAF domain of TRAF2 to the cytoplasmic domain of CD40. Together, these results suggest the zinc-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 Fig. 6. Cells were stimulated with anti-CD40 antibody and then solubilized in 1% Brij 58 or lysis buffer in which Brij 58 was replaced with 0.5% Nonidet P-40. Although Nonidet P-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 TRAFs may bind a Nonidet P-40-insoluble microdomain component. Pretreatment of cells with TPEN abolished CD40-stimulated recruitment of TRAFs to the Nonidet P-40-insoluble fraction, demonstrating that the zinc-binding domains of TRAFs are critical for their association with the insoluble fraction.

**DISCUSSION**

We have shown that engagement of CD40 results in the rapid recruitment of TRAF2 and TRAF3 from the cytosol to the membrane rafts, indicating that zinc chelation does not interfere with the binding of the TRAF domain of TRAF2 to the cytoplasmic domain of CD40. Together, these results suggest the zinc-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 Fig. 6. Cells were stimulated with anti-CD40 antibody and then solubilized in 1% Brij 58 or lysis buffer in which Brij 58 was replaced with 0.5% Nonidet P-40. Although Nonidet P-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 TRAFs may bind a Nonidet P-40-insoluble microdomain component. Pretreatment of cells with TPEN abolished CD40-stimulated recruitment of TRAFs to the Nonidet P-40-insoluble fraction, demonstrating that the zinc-binding domains of TRAFs are critical for their association with the insoluble fraction.
To determine if chelation of Zn\(^{2+}\) inhibits CD40-induced JNK activation, M12.4CD40 cells were preincubated with 10 \(\mu\)M TPEN, 10 \(\mu\)M BAPTA/AM, or 1.2\(\times\)10\(^{-4}\) M Me\(_2\)SO (DMSO) and then stimulated for 5 min at 37 \(^\circ\)C with 5 \(\mu\)g/ml isotype control mAb (–), anti-hCD40 antibody (+), or 0.6 \(\mu\)M sorbitol (S). Lysates were tested for activated JNK using GST-c-Jun-(1–79) as a substrate. Phosphorylated GST-Jun appears as a band on Western blots (C). WT, wild-type.

To determine if TPEN also inhibits the CD40-mediated raft recruitment of TRAF2, M12.4CD40 cells were preincubated with TPEN or Me\(_2\)SO and then stimulated with 1% Brij 58 or 0.5% Nonidet P-40. Detergent-soluble (Sol.) and -insoluble (Ins.) fractions were examined for TRAF2 content on Western blots (C). WT, wild-type.

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 to be TRAF-dependent. TRAF6 has also been characterized as a CD40-binding protein, yet in contrast to TRAF2 and TRAF3, it 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 zinc RING and zinc fingers of TRAF2 are not required for its binding to CD40 (7, 26). However, we found that disrupting the function of the zinc-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% Nonidet P-40, whereas the same detergent only partially solubilized TRAF2 and TRAF3.

Although our results indicate that TRAF2 and TRAF3 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 1 (36), nuclear factor eB-inducing kinase (37), germinal center kinase (38), and MEKK1, which, unlike the other kinases, may interact with the zinc-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 zinc-binding features of TRAFs in that it is largely ablated by TPEN treatment and is not evident in TRAF2\(\Delta Zn\). 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 zinc 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 observed, it seems reasonable to suggest that one or more of the zinc 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. Although 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 receptors 1 and 2, CD27, CD30, and RANK (31, 43–46), and, in each case, may mediate important interactions with microdomain-associated signaling molecules.

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