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Roles of TNF Receptor-Associated Factor 3 in Signaling to B Lymphocytes by Carboxyl-Terminal Activating Regions 1 and 2 of the EBV-Encoded Oncoprotein Latent Membrane Protein 1

Ping Xie* and Gail A. Bishop2*†§

TNF-associated factor (TRAF)3, an adaptor protein that binds the cytoplasmic domains of both CD40 and the EBV-encoded oncoprotein latent membrane protein (LMP1), is required for positive signaling by LMP1 but not CD40 in B lymphocytes. The present study further investigated how TRAF3 participates in LMP1 signaling. We found that TRAF3 mediates signaling both through direct interactions with the C-terminal activating region (CTAR)1 of LMP1 and through indirect interactions with the CTAR2 region of LMP1 in mouse B cells. Notably, our results demonstrated that the CTAR2 region appears to inhibit the recruitment of TRAF1 and TRAF2 to membrane rafts by the CTAR1 region. Additionally, the absence of TRAF2 in B cells resulted in only a modest reduction in CTAR1-mediated signals and no detectable effect on CTAR2-mediated signals. CTAR1 and CTAR2 cooperated to achieve the robust signaling activity of LMP1 when recruited to the same membrane microdomains in B cells. Interestingly, TRAF3 deficiency completely abrogated the cooperation between CTAR1 and CTAR2, supporting the hypothesis that TRAF3 participates in the physical interaction between CTAR1 and CTAR2 of LMP1. Together, our findings highlight the central importance of TRAF3 in LMP1-mediated signaling, which is critical for EBV persistent infection and EBV-associated pathogenesis. The Journal of Immunology, 2004, 173: 5546–5555.

A human gammaherpesvirus, EBV is a causative agent of infectious mononucleosis and lymphoproliferative diseases in immunosuppressed patients. It is etiologically associated with several human malignancies such as Burkitt’s lymphoma, Hodgkin’s disease, and nasopharyngeal carcinoma (1–5). Infection with EBV has the potential to produce indefinitely proliferating B lymphocytes as evidenced by the outgrowth of EBV-transformed lymphoblastoid cell lines (LCLs) (1–5). Latent membrane protein (LMP)1 is the only EBV-encoded protein that can transform rodent fibroblasts and render them tumorigenic in nude mice (4–6). Transgenic mice expressing LMP1 under the control of the mouse IgH enhancer and a VH promoter develop B cell lymphomas at an accelerated rate as they age (7). A large number of published studies indicate that LMP1 is a major contributing factor to the development of EBV-associated lymphoproliferative disease and lymphomas (1–3, 5, 8).

LMP1 is an integral membrane protein of 386 aa consisting of a short cytoplasmic N-terminal domain (aa 1–24), six transmembrane domains (aa 25–186), and a long cytoplasmic C-terminal tail (CCT) (aa 187–386) (9). The short N-terminal domain is essential for the correct insertion of LMP1 into the membrane and for the regulation of LMP1 degradation by the ubiquitin–proteasome pathway (10, 11). The six transmembrane domains of LMP1 spontaneously aggregate and oligomerize within the plasma membrane, and are responsible for the ligand-independent constitutive activation of the protein. However, the CCT appears to be the major region required for signaling per se (8, 12–15). Two domains in the CCT of LMP1 have been identified as important in LMP1 signaling: C-terminal activating region (CTAR)1 (aa 194–232) and CTAR2 (aa 351–386) (16). Adaptor proteins able to interact with CTAR1 were found to be members of the TNFR-associated factor (TRAF) family, including TRAF3, -1, -2, and -5, which are shared by members of the TNFR superfamily, such as CD40, CD30, and lymphotoxin β-R (17–20). CTAR2 has been shown to potentially bind TNFR-associated death domain protein (TRADD) and receptor-interacting protein (RIP) (21, 22). Both CD40 and LMP1 signaling recruit TRAFs to nonionic detergent-insoluble, sphingolipid-enriched membrane microdomains termed rafts (23–26). LMP1 closely mimics most of the signaling events and effector functions of CD40 in B lymphocytes, including activation of the transcription factor NF-κB and JNK, up-regulation of adhesion molecules and costimulatory molecules, as well as secretion of Abs and cytokines (reviewed in Refs. 14 and 15). However, unlike CD40, LMP1 signals to B cells in a dysregulated manner, leading to amplified and sustained B cell activation (14, 15, 24, 27).

By generating and characterizing B cell lines individually deficient in TRAF2 and TRAF3, we have recently demonstrated that CD40 and LMP1 unexpectedly used TRAF2 and TRAF3 for signaling in strikingly different ways (28, 29). In sharp contrast to the
TRAF3 and CD154 were prepared as previously described (28). BCM-10 as described (33). High Five (Hi-5) insect cells infected with wild-type (WT) baculovirus, or recombinant baculovirus expressing mouse CD154 were prepared as previously described (28).

Materials and Methods

Cell lines

The mouse B cell lines CH12.LX and M12.4.1 have been described previously (31, 32). Generation and characterization of TRAF2−/− and TRAF3−/− CH12.LX cells have been described previously in detail (28, 29), and production of TRAF1−/− CH12.LX will be reported elsewhere (B. S. Hostager and G. A. Bishop, unpublished data). Cells were cultured in RPMI 1640 supplemented with 10% FCS, 10 μM 2-ME, and antibiotics (Becton-Dickinson, Sparks, MD). High Five (Hi-5) cells were maintained in wild-type (WT) baculovirus, or recombinant baculovirus expressing mouse CD154 were prepared as previously described (28).

Abs and reagents

A hybridoma producing anti-mouse CD40 (mCD40) (clone IC10, rat IgG2a) was kindly provided by Dr. F. Lund (The Trudeau Institute, Saranac Lake, NY). A hybridoma producing anti-mouse IgE (clone EM95/3, isotype control) was a gift from Dr. T. Waldschmidt (University of Iowa, Iowa City, IA). An anti-human (h)CD40 hybridoma (clone G2B-8, mouse IgG1) and an anti-CD8 hybridoma (OKT8, isotype control of mouse IgG2a) were purchased from American Type Culture Collection (Manassas, VA). Purified MOPC-21 (isotype control of mouse IgG1) was a gift from Sigma-Aldrich (St. Louis, MO). Anti-LMP1 hybridoma (clone S12, mouse IgG2a) was a generous gift from Dr. F. Wang (Harvard University, Boston, MA). Abs were purified from hybridoma supernatants by protein A-Sepharose (Amersham) and quantitated as plaque-forming cells (PFC) per 106 viable cells recovered from replicate cultures.

Immunoblot analysis

Cell pellets of 2.5 × 106 B cells were lysed and briefly sonicated in 200 μl of lysis buffer (1% Triton X-100, 50 mM Tris (pH 8.0), 150 mM NaCl, and 50 mM β-glycerophosphate with protease and phosphatase inhibitors) supplemented with 0.5% SDS and 1% 2-ME. Aliquots of total cellular lysates were separated by SDS-PAGE and electrophoresed onto nitrocellulose membranes. JNK activation was determined by immunoblot analysis using Abs specific for phospho-JNK. Immunoblot analysis was performed with at least two different clones.

TRAF recruitment to receptors in detergent-insoluble microdomains (rafts) and immunoprecipitation

Stably transfected CH12.LX and M12.4.1 subclones (2 × 107 cells) were stimulated in a total volume of 1 ml with 10 μg of anti-hCD40 (G2B-5) or isotype control mAbs for 10 min at 37°C to induce recruitment of TRAFs to membrane rafts and allow formation of LMP1 signaling complexes. Cells were chilled on ice, lysed in 800 μl of ice-cold 1% Brij 58 lysis buffer (23), and incubated for 30 min on ice. Detergent-soluble and insoluble fractions were separated by centrifugation at 14,000 × g for 30 min. The 1% Brij 58-insoluble pellets were resolubilized in 500 μl of octylglucosylpyranoside lysis buffer (30) and sonicated, followed by a 30-min incubation on ice. The octylglucosylpyranoside lysates (raft lysates) were clarified by centrifugation at 14,000 × g for 10 min to remove the remaining insoluble samples. Samples (80 μl) of 1% Brij 58-soluble lysates as well as 50 μl of the raft lysates were retained, the remainder of the lysates were immunoprecipitated with protein G-Sepharose beads (Amersham Biosciences, Uppsala, Sweden) prearred with anti-hCD40 (G2B-5) for 3 h at 4°C. The immunoprecipitation complexes were washed four times with lysis buffer, and left in a final volume of 50 μl. Aliquots of lysates and the immunoprecipitates were separated by SDSPAGE and examined by immunoblot analysis.

Ab secretion assay

CH12.LX and its transfected subclones express surface IgM specific for phosphatidylcholine, an Ag found on the surface of SRBC (35). Enumeration of SRBC-specific-IgM-secreting cells was by direct plaque assay, as described previously (36). In brief, 1.5 × 105 cells were stimulated in 96-well plates in a total volume of 200 μl for 72 h. For reconstituted TRAF1−/− cells, TRAF3 expression was induced by preincubation with BCM-10 containing 100 μg IPTG at 37°C for 24 h, and stimuli were added for a subsequent 48 h. Ab-secreting cells were measured as cells capable of forming lytic plaques on a lawn of SRBC in the presence of complement, and quantitated as plaque-forming cells (PFC) per 106 viable cells recovered from replicate cultures.

DNAs

The mouse B cell lines CH12.LX and M12.4.1 have been described previously (31, 32). Generation and characterization of TRAF2−/− and TRAF3−/− CH12.LX cells have been described previously in detail (28, 29), and production of TRAF1−/− CH12.LX will be reported elsewhere (B. S. Hostager and G. A. Bishop, unpublished data). Cells were cultured in RPMI 1640 supplemented with 10% FCS, 10 μM 2-ME, and antibiotics (Becton-Dickinson, Sparks, MD). High Five (Hi-5) cells were maintained in wild-type (WT) baculovirus, or recombinant baculovirus expressing mouse CD154 were prepared as previously described (28).

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JNK activation analysis

CH12.LX cells (2.5 × 106) were stimulated with Hi-5 insect cells (at a ratio of B cells/Hi-5 cells of 5:1) infected with WT baculovirus or a recombinant baculovirus expressing mouse CD154 for 30 min, or with 10 μg/ml anti-hCD40 or isotype control mAbs in a total volume of 1 ml at 37°C for various time periods as indicated in the figures. Cells were immediately chilled on ice, and total cellular lysates were prepared as described above. JNK activation was determined by immunoblot analysis using Abs specific for phospho-JNK and total JNK.

DNAs

DNA constructs used to stably express hCD40LMP1 and its mutants, including hCD40CTAR1, hCD40CTAR2, hCD40PQAQA1, hCD40PQAQA2, and hCD40PQAQA3 have been described previously (24, 30). The mouse B cell lines CH12.LX and M12.4.1 have been described previously in detail (28, 29), and production of TRAF1−/− CH12.LX will be reported elsewhere (B. S. Hostager and G. A. Bishop, unpublished data). Cells were cultured in RPMI 1640 supplemented with 10% FCS, 10 μM 2-ME, and antibiotics (Becton-Dickinson, Sparks, MD). High Five (Hi-5) cells were maintained in wild-type (WT) baculovirus, or recombinant baculovirus expressing mouse CD154 were prepared as previously described (28).
NF-κB luciferase reporter assay

Stably transfected CH12.LX subclones (2.0 × 10⁷ cells) were electroporated at 200 V and 50 mS with a plasmid encoding 4× NF-κB firefly luciferase (38 μg) (a gift from Dr. E. Clark, University of Washington, Seattle, WA) and a control Renilla (pRL-null) luciferase (2 μg) reporter plasmid (Promega, Madison, WI). The 4× NF-κB luciferase construct contains four copies of the NF-κB binding sites from the promoter of the invariant chain of MHC II to drive luciferase (37), and the pRL-null was used as control for transfection efficiency. After transfection, cells were rested in medium containing 15% FCS for 5 h at 37°C. Cells were then washed and resuspended in BCM-10; an equal number of viable cells was aliquoted into 24-well plates (5.0 × 10⁵ cells/well) and stimulated overnight with 15 μg/ml anti-hCD40 or isotype control mAbs, or with Hi-5 cells (at a ratio of B cells/Hi-5 cells of 5:1) infected with WT baculovirus or a recombinant baculovirus expressing mouse CD154 in a total volume of 1.5 ml at 37°C. Cell lysates were analyzed for the firefly and Renilla luciferase activities with the Dual Luciferase Reporter Assay kit (Promega) on a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA), following the manufacturer’s protocols.

Results

LMP1 self-aggregates through its six transmembrane domains and thus is constitutively active when it is expressed on cells (5, 8, 15). It has been shown previously that only the CCT of LMP1 is required for postaggregation delivery of signals, and that LMP1 signals similarly in both human and mouse B cells (7, 8, 14, 15, 33).

To study LMP1 signaling, we have previously generated a chimeric molecule (hCD40LMP1) composed of the extracellular and transmembrane domains of hCD40 and the CCT of LMP1. This chimeric molecule signals indistinguishably from LMP1 but with controllable initiation, and like WT LMP1, its aggregation localizes the hybrid receptor to plasma membrane rafts (24, 29, 30). The extracellular domains of hCD40 and mCD40 are not cross-reactive with species-specific Abs, so hCD40LMP1 and the endogenous mCD40 can be engaged differentially (24, 29, 30). To dissect the signaling mediated by the two functional domains of the CCT of LMP1, our laboratory has also constructed two deletion mutants, hCD40CTAR1 (aa 187–241 of LMP1) and hCD40CTAR2 (aa 242–386 of LMP1), within the context of hCD40LMP1 chimeric molecule (Fig. 1A) (30). These chimeric molecules were stably transfected into CH12.LX and M12.4.1 mouse B cell lines; expression-matched clones were selected by immunofluorescence flow cytometry and used in the following study.

Differential binding of TRAFs to CTAR1 vs full-length CCT of LMP1

To understand how TRAF3 participates in LMP1 signaling, we first determined which region(s) of the CCT of LMP1 interact with TRAF3 and other adaptor proteins. Although previous studies have mapped the binding of TRAFs to the CCT of LMP1 in detail, most of those studies were conducted using yeast two-hybrid assays, in vitro pull-down experiments using GST fusion proteins, or coimmunoprecipitation analysis of LMP1 and TRAF constructs, both highly overexpressed in HEK 293T epithelial cells (17–20, 38). Verification of these findings with endogenous TRAFs in B cells is of physiological importance, because B cells are the principal targets of EBV infection in vivo. Additionally, we have found previously that a CD40 mutant (T234A) reported not to bind TRAF2 or TRAF3 when overexpressed in HEK 293T cells binds a significant amount of TRAF2 and normal amounts of TRAF3 in B cells (39). Thus, we cannot assume that overexpression experiments in epithelial cells accurately reflect the binding of normal levels of TRAFs and receptors in B cells.

To preserve the interaction between TRAFs and LMP1, most of the previous studies used 1 or 0.5% Nonidet P-40 to extract proteins before coimmunoprecipitation analysis. However, such mild lysis conditions are not sufficient to solubilize proteins assembled in membrane rafts, particularly TRAFs (23, 25), and thus may compromise the sensitivity of coimmunoprecipitation analysis.

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Our laboratory has developed an approach to better detect the recruitment of endogenous adaptor proteins to rafts upon CD40 and LMP1 signaling in B cells (23, 24, 29, 30). In this method, nonionic detergent-soluble proteins are first extracted with 1% Brij 58,
which does not disrupt rafts, and then Brij-58-insoluble proteins assembled in rafts are resolubilized with 1% Nonidet P-40 supplemented with 60 mM octylglucopyranoside and 0.1% SDS (both octylglucopyranoside and SDS ensure the solubilization of rafts) (23, 25). Brij 58-soluble and -insoluble (raft) lysates are subsequently analyzed both by direct immunoblotting and by coimmunoprecipitation followed by immunoblotting. This approach allows us to detect the recruitment of endogenous adaptor proteins to LMP1 signaling rafts, regardless of their binding avidity to LMP1 and whether the interaction is direct or indirect. Furthermore, it provides information about what relative proportion of each adaptor protein is recruited by LMP1 upon signaling. Using this approach, we have systematically determined the recruitment of TRAFs and TRADD by hCD40CTAR1 and hCD40CTAR2 in comparison with hCD40LMP1 in B cells.

Our results demonstrate that, in both M12.4.1 and CH12.LX mouse B cells, aggregation of CTAR1 recruited endogenous TRAF1, TRAF2, TRAF3, and TRAF5 to Brij-58-insoluble membrane rafts, whereas CTAR2 aggregation did not recruit any of these TRAFs (Fig. 1B). It has been shown previously that endogenous TRAF6 is translocated to membrane microdomains by LMP1 signaling in HeLa cells as visualized by immunofluorescence staining, and that TRAF6 can be coimmunoprecipitated with transfected LMP1 in HEK 293T cells (40, 41). However, no endogenous TRAF6 recruitment by CTAR1, CTAR2, or LMP1 aggregation was detected in B cells (Fig. 1B). Recruitment of TRAF6 by CD40 and coimmunoprecipitation of TRAF6 with CD40 (either endogenous mCD40 or stably transfected hCD40) upon CD40 engagement was easily detected in these cells (data not shown). Similarly, TRADD, the adaptor protein previously reported to associate with CTAR2 of LMP1 (21, 22), was not recruited by CTAR1, CTAR2, or LMP1 aggregation (Fig. 1B), although recruitment of TRADD by TNFR1 was readily detected in B cells stably transfected with hCD40TNFR1 (C). Consistent with our observations, several previous studies did not detect TRAF6 or TRADD in raft fractions with LMP1 in EBV+ LCLs or nasopharyngeal carcinoma cells (25, 26, 42).

Interestingly, engagement of hCD40LMP1 (with the full-length CCT of LMP1) recruited only ~5% of cellular TRAF1 and TRAF2. However, engagement of hCD40CTAR1 (CTAR1 only) recruited >60% of cellular TRAF1 and TRAF2 in both B cell lines (Fig. 1B). In contrast, the amounts of TRAF3 and TRAF5 recruited by the full-length CCT of LMP1 vs CTAR1 only were similar (~80% for TRAF3 and ~5% for TRAF5) (Fig. 1B). Our data extend the previous in vitro finding that TRAF1 and TRAF2 associate with a GST fusion protein of CTAR1 more efficiently than with a GST fusion protein of the full-length CCT of LMP1 (19, 38). These findings suggest that the primary sequence of CTAR1 exhibits similar high affinity for TRAF1, TRAF2, and TRAF3. However, the full-length CCT of LMP1 may interact with additional adaptor protein(s) and/or assume a different conformation, which favors the high-affinity binding of TRAF3, but hampers the binding of TRAF1 and TRAF2.

**Role of TRAFs in CTAR1 signaling**

Using B cell lines individually deficient in TRAF3 and TRAF2, we have previously shown that TRAF3 is required and indispensable for LMP1 but not CD40 signaling, whereas TRAF2 is important for CD40 but not LMP1 signaling in B cells (28, 29). Considering that the full-length CCT of LMP1 can recruit the majority of cellular TRAF3 but very little TRAF1 and TRAF2, whereas CTAR1 is able to recruit the majority of cellular TRAF1, TRAF2, and

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**FIGURE 2.** Effects of TRAF3 deficiency on IgM secretion induced by CTAR1 and CTAR2 signaling. A, CH12.LX cells stably transfected with hCD40CTAR1 were stimulated with 2 μg/ml anti-mCD40 Ab, anti-hCD40 Ab, or isotype control Abs (iso) for 72 h, and then assayed for IgM secretion. Pfc (IgM-secreting cells) per 10⁶ viable recovered cells are shown. B, Reconstituted TRAF3−/− CH12.LX cells stably transfected with hCD40CTAR1 were preincubated in the absence (−IPTG) or presence of IPTG (+IPTG) to induce the expression of TRAF3 for 24 h, and then stimulated with Abs as in A in the absence or presence of IPTG. IgM-secreting cells were enumerated as in A. C, CH12.LX cells stably transfected with hCD40CTAR2 were stimulated with Abs as in A and then assayed for IgM secretion. D, Reconstituted TRAF3−/− CH12.LX cells stably transfected with hCD40CTAR2 were treated as in B, and then assayed for IgM secretion. Values presented are the mean ± SE of replicate samples. Data are representative of five (A) or three (B–D) independent experiments. Differences in absolute numbers of Pfc in individual experiments represent day-to-day variability in sensitivity of SRBC to lysis. E, Total cell lysates were prepared from WT (+/+), TRAF3−/− (−/−), and reconstituted TRAF3−/− CH12.LX cells incubated in the absence (−IPTG) or presence of IPTG (+IPTG) for 24 h. Protein blots were sequentially immunoblotted for TRAF3, TRAF2, and hCD40.
TRAF3 (Fig. 1B), we asked whether TRAF1 and/or TRAF2 can substitute for TRAF3 in CTAR1 signaling. We thus evaluated CTAR1 signaling in WT CH12.LX cells compared with clones individually deficient in TRAF3, TRAF1, and TRAF2. Engagement of endogenous mCD40 was used as an internal control for each cell line in these experiments. Previously, we found that either hCD40CTAR1 or hCD40CTAR2 alone is defective in inducing many LMP1-mediated activation events in B cells (30). However, whereas these molecules are no longer able to induce the amplified Ab production induced by the full-length CCT of LMP1, they can induce IgM production similar to that induced by endogenous mCD40 (30). Thus, our examination of CTAR1 or CTAR2 signaling primarily focused on IgM secretion. As shown previously, in WT CH12.LX cells, CTAR1 signaling stimulated IgM secretion approximately as well as did endogenous mCD40. In sharp contrast, CTAR1-mediated IgM secretion was almost completely abolished in TRAF3−/− cells, although ligation of endogenous mCD40 induced a higher level of IgM secretion in the absence of TRAF3 (Fig. 2A), due to the inhibitory effect of TRAF3 on CD40-induced IgM secretion (29). To verify that this resulted from the loss of TRAF3, we examined TRAF3−/− CH12.LX cells stably reconstituted with an IPTG-inducible TRAF3. Induction of normal endogenous levels of TRAF3 expression completely restored IgM secretion in response to CTAR1 signaling, while decreasing IgM secretion mediated by mCD40 signaling (Fig. 2, B and E). Together, these data indicate that TRAF3 is required and essential for CTAR1-mediated IgM secretion in B cells. Interestingly, CTAR1-mediated IgM secretion was modestly reduced in TRAF1−/− and TRAF2−/− CH12.LX cells (Fig. 3A), suggesting that TRAF1 and TRAF2 may also contribute to CTAR1 signaling, but play a much lesser role than TRAF3.

**FIGURE 3.** Effects of TRAF1 or TRAF2 deficiency on IgM secretion induced by CTAR1 and CTAR2 signaling. A, CH12.LX cells stably transected with hCD40CTAR1 were stimulated with 2 μg/ml anti-mCD40 Ab, anti-hCD40 Ab, or isotype control Abs (iso) for 72 h, and then assayed for IgM secretion. Pfc (IgM-secreting cells) per 10^6 viable recovered cells are shown. B, CH12.LX cells stably transected with hCD40CTAR2 were stimulated with Abs as in A, and then assayed for IgM secretion. Values presented are the mean ± SE of replicate samples. Data are representative of four (A) or three (B) independent experiments. C, Total cell lysates were prepared from WT, TRAF1−/− (T1−/−), and TRAF2−/− (T2−/−) CH12.LX cells. Protein blots were sequentially immunoblotted for TRAF2, TRAF1, TRAF3, and hCD40. NS, Nonspecific band. Comparable level of expression of the transfected hCD40CTAR1 or hCD40CTAR2 between WT and TRAF1−/− or TRAF2−/− cells was verified with this assay.

TRAF3 is required for the cooperation between CTAR1 and CTAR2

It has been previously shown in the Jurkat T cell line that CTAR1 and CTAR2 cooperate in signaling and that such cooperation requires physical association (either direct or indirect) within the same hetero-oligomeric complex (43). To test potential cooperation between CTARs in B cells, the principal target of EBV infection, our laboratory previously generated and examined B cell lines stably expressing hCD40CTAR1 and HLA-A2CTAR2 (composed of the extracellular and transmembrane domains of HLA-A2 and aa 242–386 of LMP1) (30). Simultaneous engagement of hCD40CTAR1 and HLA-A2CTAR2 suggested interactions between the CTARs, but could not completely restore signaling to levels seen with the full-length CCT of LMP1 (30). It is likely that hCD40CTAR1 and HLA-A2CTAR2 expressed by these B cells are recruited to distinct membrane microdomains by their corresponding agonistic Abs and thus may not be brought in sufficient proximity to allow physical interaction between CTAR1 and CTAR2. We thus generated B cell lines stably cotransfected with hCD40CTAR1 and hCD40CTAR2 as described in Materials and Methods. Previous work has shown that the full-length CCT of LMP1 provides a strongly amplified activation signal to B cells, compared with that delivered by the endogenous mCD40 (24, 30). As shown in Fig. 4A, engagement of either CTAR alone only stimulated IgM secretion to a level similar to that induced by endogenous mCD40, as in Fig. 2. However, coengagement of CTAR1 and CTAR2 with the same agonist restored the signaling activity of LMP1 to ~4-fold above that of activation induced via endogenous mCD40. These results indicate that CTAR1 and CTAR2 need not be located in the same linear sequence to achieve the robust signaling activity of LMP1, if they are recruited to the same...
CTAR1 and CTAR2 cooperate to stimulate IgM secretion in WT but not TRAF3−/−. CH12.LX B cells. A, WT CH12.LX cells stably transfected with hCD40LMP1 (hCD40LMP1), hCD40CTAR1 (CTAR1), hCD40CTAR2 (CTAR2), or hCD40CTAR1 and hCD40CTAR2 (CTAR1&CTAR2) were stimulated with 2 μg/ml anti-mCD40 Ab, anti-hCD40 Ab, or isotype control Abs (iso) for 72 h, and then assayed for IgM secretion. Pfc (IgM-secreting cells) per 10⁶ viable recovered cells are shown. B, TRAF3−/− CH12.LX cells stably transfected with hCD40LMP1 (LMP1), hCD40CTAR1 (CTAR1), hCD40CTAR2 (CTAR2), or hCD40CTAR1 and hCD40CTAR2 (CTAR1&CTAR2) were stimulated with Abs as in A, and then assayed for IgM secretion. C, WT and TRAF3−/− CH12.LX cells stably cotransfected with hCD40CTAR1 and hCD40CTAR2 were stimulated with Abs as in A and then assayed for IgM secretion. Values presented are the mean ± SE of replicate samples. Data are representative of three (A and B) or four (C) independent experiments.

This information, together with our present findings, suggests the existence of CTAR2-interacting protein(s) that directly or indirectly associate with TRAF3 (and may change the conformation of the full-length CCT of LMP1 to impede the binding of TRAF1 and TRAF2), and contribute to LMP1 signaling. Candidate proteins may be novel members of the TRAF family or TRADD-like molecules. This hypothesis predicts that mutations of PXQXT/S or YYD motifs, which eliminate the binding of CTAR2-interacting TRAF-like or TRADD-like molecule(s), may also relieve the spatial hindrance and thus restore the high affinity binding of LMP1 to TRAF1 and TRAF2. To assess this possibility, we examined the effects of mutations of PXQXT/S and YYD motifs of LMP1 on TRAF binding.

As shown in Fig. 6B, mutation of the first PQQT motif to AQAAT nearly eliminated the recruitment of TRAF3, TRAF1, TRAF2, and TRAF5. These data extend the previous finding that the first PQQT motif of LMP1 mediates the direct binding of these TRAFs (19, 20, 38) to interactions with endogenous TRAFs in B lymphocytes. Mutation of the second PPQLT motif to APALT did not affect the binding of TRAF3, TRAF1, and TRAF5, but slightly increased the recruitment of TRAF2, to ~1.5-fold of the amount recruited by the WT LMP1 CCT. Mutation of the third PVQLS motif to AVALS or mutation of YYD motif to AAA did not alter the binding of TRAF3 and TRAF5, but modestly enhanced the binding of TRAF2 and TRAF1 to ~2- and 1.5-fold of WT LMP1-bound level, respectively. TRAF6 and TRADD were not detectably recruited by any of the mutants or WT LMP1. Hence, mutation of the second or third PXQXT/S motif, or the YYD motif individually was not sufficient to allow the full-length CCT of LMP1 to bind TRAF1 or TRAF2 as well as the hCD40CTAR1 molecule does, but did modestly increase their binding. Taken together, these data suggest that these motifs may be involved in the interaction between CTAR1 and CTAR2, but also indicate the likelihood that additional structural motif(s) are required.

Discussion

We have recently reported that TRAF3 is required for LMP1- but not CD40- mediated B cell activation (29). The experiments described here sought to further elucidate how TRAF3 participates in LMP1 signaling in B cells. We found that TRAF3 mediated

membrane microdomains, suggesting that efficient physical interaction between CTAR1 and CTAR2 is required for optimal LMP1 signaling in B cells. Our finding that TRAF3 may indirectly regulate CTAR2 signaling prompted us to further test whether TRAF3 is required for the physical interaction between CTAR1 and CTAR2. Characterization of TRAF3−/− B cells stably cotransfected with hCD40CTAR1 and hCD40CTAR2 revealed that the cooperative signaling effects of CTAR1 and CTAR2 on IgM secretion observed in WT cells were completely abrogated in TRAF3−/− B cells (Fig. 4, A and C).

We also investigated the potential cooperation between CTAR1 and CTAR2 in mediating activation of JNK and NF-κB, two essential proximal signaling events mediated by LMP1. In WT CH12.LX B cells, neither CTAR alone was able to induce obvious JNK activation, but signaling through both CTAR1 and CTAR2 fully reconstituted JNK activation to levels induced by the full-length CCT of LMP1 (Fig. 5A). Cooperation between CTAR1 and CTAR2 was also seen in NF-κB activation in WT B cells (Fig. 5B). Interestingly, cooperative effects of CTAR1 and CTAR2 on JNK activation and NF-κB activation were diminished by TRAF3 deficiency (Fig. 5). Together, our findings support the hypothesis that TRAF3 plays an important role in the interaction between CTAR1 and CTAR2.

Effects of mutations of PXQXT/S and YYD motifs on TRAF binding

There are three PXQXT/S motifs (potential TRAF binding motifs) in LMP1, the first located in CTAR1, the second located in the intermediate region between CTAR1 and CTAR2, and the third located in CTAR2. The potential TRADD (or RIP) binding motif YYD is located at the C terminus (aa 384–386) (Fig. 6A). It was shown previously that mutation of the first PXQXT/S motif alone leads to defective CTAR1 signaling, whereas mutation of the third PXQXT/S motif or the YYD motif alone results in defective CTAR2 signaling (21, 30, 44). These findings suggest that the third PXQXT/S motif and the YYD motif of LMP1 may mediate the binding of CTAR2-interacting adaptor proteins. However, neither any known TRAF molecule nor TRADD was detectably recruited by CTAR2 or the full-length CCT of LMP1 in B cells (Fig. 1B). It is known that TRAFs can form heterodimers via their TRAF-C domains and that TRAF2 can associate with TRADD (8, 15, 45).
CTAR1 signaling by directly interacting with CTAR1 through the first PQQAT motif. Notably, our data indicated that TRAF3 also regulated CTAR2 signaling indirectly. Reinforcing a previous finding that CTAR1 and CTAR2 cooperate in signaling through physical interaction in Jurkat T cells (43), we observed that CTAR1 and CTAR2 cooperated to achieve the robust signaling activity of LMP1 if they are recruited to the same membrane microdomains in B cells. The finding that TRAF3 deficiency completely abrogated the cooperation between CTAR1 and CTAR2 is consistent with a model wherein TRAF3 participates in the physical interaction between CTAR1 and CTAR2 of LMP1. Our findings highlight the essential role of TRAF3 in LMP1-mediated signaling, which is critical for EBV persistent infection and EBV-associated pathogenesis.

It has been widely believed that TRAF2 is critical for mediating LMP1 signals both directly by binding to CTAR1 and indirectly by binding to CTAR2 through association with TRADD (see reviews in Refs. 5 and 8). These conclusions were based on studies in which overexpression of WT or dominant-negative TRAF2 was used, mostly with HEK 293 cells, and seemed reasonable in light of the evidence that TRAF2 is a powerful activator of positive signals by several members of the TNFR superfamily (45–47). However, increasing evidence argues against this previous model to explain the effects of LMP1 on B lymphocytes. First, very little (~5%) cellular TRAF2 is associated with LMP1 in stably transfected B cells or EBV-transformed B lymphocytes (19, 25, 29). Second, LMP1-mediated NF-κB activation, JNK activation, and IgM secretion are intact in TRAF2−/− B cells (29). Third, CTAR2-induced IgM secretion is normal in TRAF2−/− B cells (Fig. 3B). Fourth, LMP1-stimulated JNK activation is unaffected in TRAF2−/− mouse embryonic fibroblasts (MEFs) (41). Finally, LMP1-induced NF-κB activation is nearly normal in TRAF2 and TRAF5 double-knockout MEFs (48). Thus, the preponderance of accumulating evidence indicates that TRAF2 does not play essential, nonredundant roles in key LMP1-mediated activation events in B cells and fibroblasts.

However, it is fascinating that a truncated version of LMP1, CTAR1, binds much more efficiently to TRAF1 and TRAF2 than the full-length CCT of LMP1 (Fig. 1B; Refs. 19 and 38). The majority (~60%) of cellular TRAF1 and TRAF2 was recruited by CTAR1 in B cells, and CTAR1-mediated IgM secretion was moderately decreased in TRAF1−/− or TRAF2−/− B cells (Figs. 1B and 3A), suggesting that TRAF1 and TRAF2 may contribute to signaling by this truncated version of LMP1. These findings have important pathological relevance, given the evidence that naturally
occurring truncated versions of LMP1 exist (49, 50). In EBV-transformed LCLs and EBV/H11001 Burkitt’s lymphoma cell lines, one natural cleavage in LMP1 removes 145 aa from the C terminus resulting in an N-terminal product (aa 1–241) aggregated in the plasma membrane (49), which should have similar TRAF-binding properties to CTAR1. This truncated version of LMP1 (aa 1–241) has been shown to be sufficient for B cell transformation, whereas CTAR2 is important for long-term outgrowth of EBV-transformed B cells (12). Another truncated version of LMP1 (lyLMP1, aa 129–386), which only has the fifth and sixth transmembrane domains and the CCT of LMP1 and thus cannot oligomerize, is synthesized in the late lytic cycle of EBV infection (50). lyLMP1 is packed in the EBV virion and has been proposed to play a role in the initial stage of infection and during the lytic cycle (51). It is conceivable that different versions of LMP1 may not only have different signaling capabilities and functional roles, but also differentially alter the stoichiometry of various TRAF molecules available for cellular receptors of the TNFR superfamily. Full-length LMP1 would sequester most cellular TRAF3, whereas the CTAR1 truncated version of LMP1 may limit the availability of TRAF3, TRAF1, and TRAF2 for other receptors. In contrast, lyLMP1 may not sequester any TRAFs. Therefore, we can envisage that different LMP1 proteins not only usurp TRAFs to transduce signals, but also indirectly modulate the function of normal receptors of the TNFR superfamily, leading to B cell immortalization, transformation, or lysis.

We consistently detected a small amount of cellular TRAF5 (~5%) recruited by CTAR1 or the full-length CCT of LMP1 upon signaling (Figs. 1B and 6B). However, the functional roles of TRAF5 in LMP1-mediated signaling remain to be determined. Although it has been previously shown that overexpression of dominant-negative TRAF5 abrogates CTAR1-mediated NF-κB activation (5), it was recently found that LMP1-induced NF-κB activation is nearly normal in TRAF2 and TRAF5 double-knockout MEFs (48). Interestingly, recent studies reported that both JNK activation and NF-κB activation mediated by LMP1 signaling are severely impaired in TRAF6/H11002/MEFs, suggesting an indispensable role for TRAF6 in LMP1 signaling in fibroblasts (41, 48). It has been shown previously that endogenous TRAF6 is translocated to membrane microdomains by LMP1 signaling in HeLa cells as visualized by immunostaining and can be coimmunoprecipitated with transfected LMP1 in HEK 293T cells (40, 41). However, no TRAF6 recruitment by CTAR1, CTAR2, or LMP1 signaling was detected in B cells (Fig. 1B). Similarly, direct interaction between TRAF6 and LMP1 could not be detected by yeast two-hybrid assays or in vitro pull-down assays using GST fusion proteins (41, 48). In this context, it would be interesting to investigate whether TRAF6 is able to regulate LMP1 signaling indirectly in B cells. B cell lines individually deficient in TRAF5 and TRAF6 should be valuable in elucidating the functional roles of these TRAFs in LMP1 signaling.

FIGURE 6. Recruitment in B cells of TRAFs by the CCT of LMP1 with mutated PXQXT/S or YYD motifs. A, Schematic representations of hCD40LMP1 (WT), hCD40PQAA1 (PQAA1), hCD40PQAA2 (PQAA2), hCD40PQAA3 (PQAA3), and hCD40YYD3A (YYD3A). Sequences of the three potential TRAF binding motifs PXQXT/S and the TRADD binding motif YYD in WT LMP1 and the mutants are shown. B, M12.4.1 cells stably transfected with hCD40LMP1 (WT), hCD40PQAA1 (PQAA1), hCD40PQAA2 (PQAA2), hCD40PQAA3 (PQAA3), or hCD40YYD3A (YYD3A) were stimulated with 10 μg/ml anti-hCD40 Ab to trigger signaling through these chimeric receptors for 10 min. Detergent soluble (S) and insoluble raft (I) lysates were prepared. Ninety percent of the lysates were incubated with anti-hCD40 Ab (G28-5) to immunoprecipitate the chimeric receptors. The lysates and immunoprecipitates were analyzed by immunoblotting for TRAF2, TRAF1, TRAF3, TRAF5, TRAF6, TRADD, and hCD40LMP1. NS, Nonspecific band. Results shown are representative of two independent experiments.
It is unclear what adaptor protein(s) is directly responsible for the CTAR2-induced IgM secretion. TRADD has been generally considered as the critical adaptor protein directly interacting with CTAR2 through the C-terminal YYD motif (reviewed in Refs. 5 and 8). Interaction between TRADD and CTAR2 was initially identified by yeast two-hybrid analysis, and verified by coimmunoprecipitation analysis in HEK 293 cells and EBV-transformed LCLs (21, 22). However, we could not detect any recruitment of TRADD by CTAR2 or the full-length CCT of LMP1 upon signaling in B cells, although we easily detected recruitment of TRADD by TNFR1 signaling (Fig. 1, B and C). Consistent with our observations, several other studies could not detect TRADD in LMP1 signaling rafts in LCLs and nasopharyngeal carcinoma cells (25, 26, 42). One explanation for this discrepancy is that TRADD associates with LMP1 only in the detergent-soluble fraction but not in the detergent-insoluble raft fraction, because the initial coimmunoprecipitation analysis of TRADD and LMP1 in EBV-transformed LCLs was performed with Brij-58-soluble lysates. However, coimmunoprecipitation of TRADD with LMP1 or CTAR2 was detected neither in Brij-58-soluble lysates nor in Brij-58-insoluble raft lysates in B cells in the present study (Figs. 1B and 6B), and Rothenberger et al. (42) could not coimmunoprecipitate TRADD with LMP1 in HEK 293 cells using Brij-58 or CHAPS lysis buffer. A recent study using small interfering RNA for TRADD with LMP1 in HEK 293 cells using Brij-58 or CHAPS, and Rothenberger et al. (42) could not coimmunoprecipitate soluble raft lysates in B cells in the present study (Figs. 1, B and C). Therefore, we conclude that the discrepancy between our result and these previous reports is due to the detergent solubility of TRADD.

It has been shown that the CTAR2-induced signaling motif of LMP1 in the cytoplasmic domain of LMP1 is sufficient to activate NF-κB and JNK activation. However, mutations of the YYD motif and/or its adjacent PVQLS motif at the C terminus of LMP1 have been consistently found to cause defective CTAR2 signaling in different cell types (21, 30, 40, 44). For example, although mutation of the second PVQLS motif to APALT only slightly affected LMP1-included NF-κB and JNK activation, mutation of the third PVQLS motif to AVALS resulted in defective NF-κB and JNK activation as well as reduced IgM secretion in B cells (30). These findings indicate that the third PVQLS motif and YYD motif in the C terminus of LMP1 may mediate the interaction of alternative adaptor protein(s) critical for CTAR2 signaling. Although RIP has been shown to potentially interact with the YYD motif of LMP1, it has not been implicated in LMP1 signaling, because RIP deficiency does not affect LMP1 signaling in Jurkat T cells or MEFs (22, 41). Hence, it is likely that as-yet-unidentified adaptor protein(s) mediate CTAR2 signaling through binding to the third PVQLS motif and YYD motif of LMP1. Such adaptor protein(s) may directly or indirectly associate with TRAF3 to bridge the physical interaction between CTAR1 and CTAR2 either intramolecularly or cross-molecularly. It is also possible that TRAF6 may contribute to CTAR2 signaling indirectly, a question of interest for future research. Complete delineation of the profile of factors assembled in the LMP1 signaling rafts, either directly bound to LMP1 or indirectly recruited by adaptor proteins or structural components, will be key to gain a better understanding of the molecular mechanisms of LMP1 signaling.

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References


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