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Regulation of TRAF2 Signaling by Self-induced Degradation*

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Receptors belonging to the tumor necrosis factor receptor (TNF-R) family utilize cytoplasmic adapter proteins called TNF-R-associated factors (TRAFs) as key elements in their signaling pathways. However, it is not yet clear how individual TRAFs regulate signaling by this large and growing receptor family. Signaling via the TNF-R family member CD40 has recently been shown to result in recruitment of TRAF2 to plasma membrane detergent-resistant microdomains (lipid rafts) as well as to subsequently initiate TRAF2 degradation. As TRAF2 associates with most members of the TNF-R family, we wished to determine how this degradation occurs. We show here that CD40-mediated TRAF2 degradation requires the zinc-binding RING domain of TRAF2 and is preceded by TRAF2 ubiquitination, suggesting that the TRAF2 RING may promote ubiquitination although the RING itself is not a target of ubiquitination. Several approaches show that ubiquitination and proteasomal activity are integral to TRAF2 degradation, and inhibition of this process potentiates CD40 signaling.

The TNF-R[1] family is a large and growing group of receptors that make important contributions to both cellular activation and programmed cell death (reviewed in Refs. 1 and 2). A key feature in the signal transduction pathways of members of this family is their association with intracellular adapter proteins called TRAFs. Although loss- and gain-of-function experiments have demonstrated the importance of TRAFs in mediating signaling by TNF-R members (3), it is still unclear how individual TRAFs regulate signaling pathways or how they themselves are regulated. The TRAF molecule that has received the greatest attention to date is TRAF2, one of the two prototypic TRAFs first isolated as associating with TNF-R2 (4) and subsequently found to interact with TNF-R1, CD40, (5), and a wide variety of other TNF-R family molecules. Additionally, TRAF2 binds the cytoplasmic domain of latent membrane protein 1 (LMP1) (6), an Epstein-Barr virus-encoded transforming protein that mimics CD40 signals to B cells (7, 8).

In an effort to understand the mechanism of the transforming effects of LMP1 on B cells, we recently performed a direct comparison of the signal transduction pathways of CD40 and LMP1. A striking result of this analysis was that although both CD40 and LMP1 recruit TRAF2 to lipid rafts in the plasma membrane, CD40, but not LMP1, subsequently promotes TRAF2 degradation, and this difference is associated with sustained and amplified signaling by LMP1 (9). CD40 stimulation of normal resting mouse splenic B cells also induces TRAF2 degradation.[2] These findings suggest that TRAF2 degradation may be an important component of normal signaling by TNF-R family molecules, an idea supported by the demonstration that CD30 signaling also stimulates a loss of cellular TRAF2 (10).

The present work was instigated to better understand the molecular mechanisms by which TRAF2 degradation is mediated. The TRAF2 molecule is composed of an N-terminal RING domain, a series of zinc fingers, and a conserved C-terminal TRAF domain that contains the region for binding to TNF-R family members and for promoting association of TRAF2 with itself and other TRAF molecules (4). Interestingly, certain other signaling molecules that contain RING domains are reported to promote their own degradation or degradation of other proteins with which they interact, by stimulating ubiquitination (11, 12). This ubiquitination and degradation is dependent upon the RING domain of these molecules, which may serve as an E3 ubiquitin protein ligase, although this specific role has thus far only been demonstrated *in vitro*. As CD40-induced TRAF2 degradation requires the activity of the 26 S proteasome (9) (which classically degrades ubiquitinated proteins) and TRAF2 contains a RING domain, we wished to determine whether CD40 initiates TRAF2 degradation by promoting its ubiquitination in a RING-dependent manner. Data presented here support this hypothesis and additionally indicate that blocking TRAF2 ubiquitination or proteasomal degradation potentiates initiation of CD40 signals.

EXPERIMENTAL PROCEDURES

Cells—The mouse B cell line M12.4.1 (13) was maintained in RPMI 1640 medium with 10% fetal calf serum, 10 μM 2-mercaptoethanol, and antibiotics. Transfected B cell subclones were maintained in culture medium supplemented with 400 μg/ml G418 sulfate (Invitrogen); some subclones were also grown in 500 μg/ml zeocin (Invitrogen). Cell lines containing inducible wild-type mTRAF2 or inducible mTRAF2ΔRING have been previously described (14, 15). Subclones stably expressing HA-tagged ubiquitin were prepared in our laboratory, by transfecting M12.4.1 with a plasmid expressing ubiquitin driven by an RSV promoter (see below). The Chinese hamster ovary (CHO) cell lines E36 and

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[1] The abbreviations used are: TNF-R, tumor necrosis factor receptor; TRAF, TNF-R-associating factor; LMP, latent membrane protein; CHO, Chinese hamster ovary; WT, wild type; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; IPTG, isopropyl-β-D-thiogalactopyranoside; ts, temperature-sensitive; JNK, Jun N-terminal kinase; RSV, Rous sarcoma virus; HA, hemagglutinin; Ab, antibody.

[2] K. D. Brown and G. A. Bishop, unpublished results.

ts-20 were a kind gift from Dr. Alan Schwartz (Washington University, St. Louis, MO) and were transfected with human CD40 (hCD40) and TRAF2 as previously described (16). CHO transfectants were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum, non-essential amino acids, antibiotics and 400 μg/ml G418 sulfate, and/or 500 μg/ml zeocin. Sf9 insect cells infected with WT baculovirus (Sf9) or recombinant virus expressing mCD154 (Sf9-mCD154) have been previously described (17).

Antibodies—Biotinylated mouse anti-FLAG (Bio-M2), mouse anti-FLAG (M2), and mouse IgG_1 isotype control mAb (MOPC-21) were purchased from Sigma Chemical. Anti-mouse actin (C4) was purchased from Chemicon (Temecula, CA). Rabbit anti-TRAF2 (C-20, H-249), anti-TRAF1 (N-19), and rabbit anti-IκBα (FL) Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody specific for the HA epitope tag was purchased from Covance (Richmond, CA). Goat anti-rabbit horseradish peroxidase (HRP) and goat anti-mouse HRP Abs were purchased from Bio-Rad (Hercules, CA). Anti-p38 MAPK (catalog no. 9212) and anti-phospho-p38 MAPK (catalog no. 9211) were purchased from Cell Signaling Technology (Beverly, MA). Anti-human CD40 mAb (G28–5, mouse IgG_1) was produced in our laboratory by a hybridoma purchased from American Type Culture Collection (ATCC, Manassas, VA). A polyclonal sheep anti-hCD40 Ab was produced in our laboratory (15). The following antibodies were produced from hybridomas provided as generous gifts from the indicated individuals: anti-mouse CD40 (1C10, rat IgG_{2a}) from Dr. Frances Lund (Trudeau Institute, Saranac Lake, NY), and anti-mouse IgE (EM95.3, rat IgG_{2a} isotype control) from Dr. Thomas Waldschmidt (University of Iowa, Iowa City, IA).

Chemicals—Octylglucoside, protein G-Sepharose, and extravidin peroxidase were purchased from Sigma Chemical. MG132 proteasome inhibitor was purchased from Calbiochem (La Jolla, CA). Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Amresco (Solon, OH).

DNA Constructs—The generation of the hCD40-LMP1 chimera and WT-hCD40 constructs has been previously described (9). Mouse TRAF2 constructs expressed inducibly contained LacR binding sites upstream of the cDNA insertion site in the plasmid and were transfected into subclones of M12.4.1 stably constitutively expressing LacR. Expression is induced by incubation in the presence of IPTG, as described in detail previously (7, 14). Inducible constructs of wild-type mTRAF2 and the mutant mTRAF2ΔRING were produced as described (14). C34S, K21R, and K31R/K38R mutants of mTRAF2 were generated utilizing a point mutagenesis kit from Stratagene (La Jolla, CA) according to the manufacturer's directions. The HA-ubitquitin construct was produced by subcloning HA-ubiquitin from a plasmid obtained from Dr. Dirk Bohmann (European Molecular Biology Laboratory, Heidelberg, Germany) (18) into pRSV.zeo. This expression vector was produced in our laboratory by replacing the cytomegalovirus promoter of pcDNA3.zeo (Invitrogen) with the RSV promoter from the pRSV.5(neo) plasmid (19).

Transfections—Transfection of M12.4.1 cells by electroporation has been previously described (16). For these studies, the M12.Lac subclone expressing the bacterial LacR (7) was used. Drug-resistant subclones containing inducible TRAF2 molecules or constitutively expressed HA-ubiquitin were examined by intracellular staining for FLAG and/or HA utilizing a FACScan flow cytometer (BD PharMingen). Temperature-sensitive (ts-20) and parent (E36) CHO cell lines were initially transfected with wild-type hCD40 cDNA containing a zeocin resistance marker, and zeocin-resistant clones of ts-20 cells expressing hCD40 were isolated by subcloning. High hCD40-expressing E36 cells were isolated by fluorescence-activated cell sorting. Both ts-20 and E36 cell lines expressing hCD40 were then transfected with a FLAG-mTRAF2 construct containing a neomycin resistance gene. E36 and ts-20 cell lines express similar levels of hCD40 (data not shown).

TRAF Degradation Assays—In earlier studies of TRAF function in B cells, we found that these cells do not express large amounts of endogenous TRAF proteins. Thus, to create as biologically valid a signaling system as possible, we developed a method to stably and inducibly express transfected TRAFs in cell lines (14), as described above. Using this method, as previously described (14), levels of transfected TRAFs only slightly above endogenous levels are routinely induced, rather than the gross overproduction often obtained in many transiently transfected epithelial and fibroblast cell lines. Degradation of inducible wild-type or mutant FLAG-tagged TRAF2 was stimulated following 18 h of incubation with 0.1 mM IPTG to induce expression of tagged TRAF2 (10^7 cells). After IPTG incubation, cells were stimulated for 120 min with 1 μg/ml anti-CD40 or isotype control mAbs. Following stimulation in experiments with cells inducibly expressing TRAF2ΔRING, total lysates were prepared as described above. This mutant TRAF is of a different molecular weight than endogenous TRAF2, and the two can therefore be discriminated by their sizes. Lysates were separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blotting. Anti-FLAG immunoprecipitations were performed to permit examination of degradation of point mutants of mouse TRAF2, because mutant and endogenous TRAF2 are the same molecular weight, and anti-FLAG blotting of total cell lysates is too nonspecific to permit definitive identification of mutant TRAF2. Cells were lysed and sonicated in octylglucoside buffer, and immunoprecipitations were performed as previously described (20). Dependence of TRAF2 degradation upon an intact ubiquitination system was examined by plating 5×10^6 hCD40 + TRAF2-transfected CHO cells of the parent cell line E36 or the ts-20 cell line containing temperature-sensitive E1 enzyme for 18 h in 35-mm dishes to allow adherence. Cells were then preincubated for 2 h at either 37 °C (permissive) or 42 °C (non-permissive) temperatures and stimulated for 2 h with either 1 μg/ml anti-hCD40 (G28–5) or mouse IgG_1 isotype control Ab (MOPC-21). Following stimulation cells were removed from the plate using 0.1 mM EDTA and pelleted, lysed, and sonicated in octylglucoside buffer. Immunoprecipitations were performed as described previously (20).

TRAF2 Modification Assays—M12.4.1 cells stably transfected with HA-tagged ubiquitin and IPTG-inducible, FLAG-tagged mTRAF2 were incubated overnight in the presence or absence of 100 μM IPTG. Cells (1×10^7 in 200 μl of culture medium) were stimulated for 15 min at 37 °C with Sf9 or Sf9-mCD154. The insect cells were used as stimulus in these assays to avoid inadvertent precipitation of unmodified TRAF2 due to the ability of stimulating anti-CD40 Abs to bind protein G. Cells were lysed in 400 μl of lysis buffer (0.1% SDS, 1.0% Triton X-100, 150 mM NaCl, 20 mM Tris, pH 7.5, and protease inhibitors). Immunoprecipitations were performed as above, and precipitates were subjected to SDS-PAGE and Western blotting.

JNK Activation Assays—To measure c-Jun kinase (JNK) activation in the presence or absence of proteasomal inhibition, cells were preincubated in the presence or absence of 50 μM MG132 (21) and then stimulated for 15, 30, or 60 min with 2 μg/ml anti-CD40, or 60 min with 2 μg/ml isotype control mAbs. Cell lysates were prepared and JNK activity measured as previously described (22). Reactions were separated by SDS-PAGE and phosphorylated c-Jun visualized by autoradiography of dried gels.

RESULTS

CD40-induced TRAF2 Degradation Requires an Intact TRAF2 RING Domain—Previous studies have indicated that other RING-containing proteins can undergo ubiquitination and degradation in a manner dependent upon their RING domains (23). If the TRAF2 RING serves this function, its removal should block TRAF2 degradation. To test this prediction, we used a previously described TRAF2 truncation mutant lacking the RING domain (T2ΔRING); this mutant binds CD40 similarly to WT TRAF2 (14). FLAG epitope-tagged mutant TRAF2 molecules were inducibly and stably expressed in the CD40-responsive mouse B cell line M12.4.1. A minimum of two subclones expressing each molecule were tested (not shown). Stimulation of the B cells with agonistic anti-mCD40 mAb led to degradation of endogenous TRAF2 as early as 10 min following stimulation with only $\sim$ 10% of the cellular TRAF2 remaining after 2 h of stimulation (Fig. 1, *A* and *D*). In contrast, however, CD40 stimulation did not induce appreciable degradation of inducibly expressed T2ΔRING (Fig. 1, *B* and *D*). This failure to induce degradation of the mutant is not due to the presence of the epitope tag on the molecule, as WT FLAG-tagged TRAF2 is degraded by CD40 stimulation (Figs. 2 and 4, *bottom*). These data thus demonstrate a specific requirement for the RING domain in CD40-induced TRAF2 degradation.

We next considered two possible roles (not mutually exclusive) that the TRAF2 RING could play in CD40-induced degradation. First, as discussed earlier, the RING structure itself could serve to promote ubiquitin ligation. However, there are also three lysine residues present in this domain. It was therefore possible that ubiquitination targets one or more of these residues, and the removal of the entire RING abrogates degradation because the target for ubiquitination has been removed.

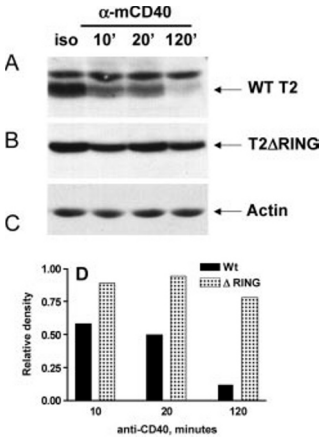

FIG. 1. **CD40-induced TRAF2 degradation requires the TRAF2 RING domain.** M12.4.1 B cells stably inducibly expressing TRAF2ΔRING were incubated in the presence or absence of 0.1 mM IPTG for 18 h to induce expression of the mutant. Cells were stimulated with either 2 μg/ml mouse isotype control Ab (*iso*) for 120 min or with anti-mCD40 Ab (*α-mCD40*) for 10, 20, or 120 min. Total cell lysates were prepared as described under "Experimental Procedures" and were separated by SDS-PAGE, transferred to nitrocellulose, and blotted for TRAF2 (*A* and *B*) or actin as a loading control (*C*). Values presented in *D* were obtained by performing densitometry on bands shown in *A–C*. Density as a proportion of isotype control is presented, normalized to the value of the actin band. Data are representative of three independent experiments.

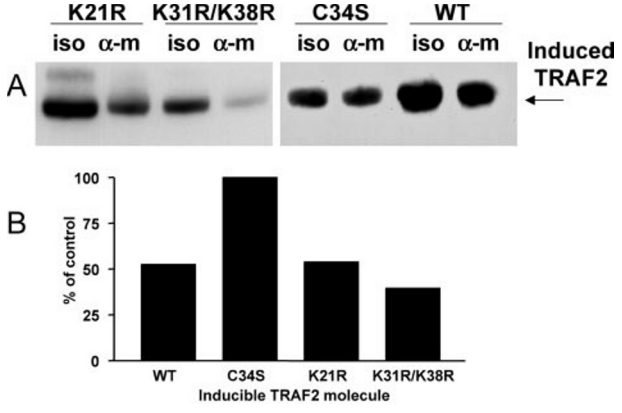

FIG. 2. **Requirement for an intact TRAF2 RING finger but not RING domain lysines in CD40-induced TRAF2 degradation.** *A*, M12.4.1 cells stably inducibly expressing FLAG-WT TRAF2 or the TRAF2 point mutants C34S, K21R, and K31R/K38R were induced with IPTG and stimulated as described in the legend to Fig. 1. Following stimulation cells were lysed and immunoprecipitated with α-FLAG. FLAG precipitates were separated by SDS-PAGE, transferred to nitrocellulose, and blotted for TRAF2. Lysates of samples precipitated in *A* were blotted with anti-actin to control for protein added to precipitation beads. Samples showed equal protein amounts (not shown). Data are representative of four independent experiments using two clones each expressing the indicated mutants. *B*, quantitation of information presented in *A*. Values are from densitometric analysis of TRAF2 bands from anti-mCD40-stimulated samples, expressed as percent of isotype control-stimulated samples.

To test both these possibilities, we constructed a series of TRAF2 point mutants. Two of these mutants remove between them all three of the lysine residues in the TRAF2 RING domain (K21R, K31R/K38R). We also constructed a cysteine to serine point mutant at the first cysteine residue in the RING (C34S). This mutation has been previously reported to disrupt RING structure (12). All mutants were stably and inducibly expressed in M12.4.1 B cells and all bind CD40 similarly to WT TRAF2 (not shown). Stimulation of B cells with anti-mCD40 led to degradation of transfected WT mouse TRAF2, as well as both K21R and K31R/K38R TRAF2 mutants (Fig. 2*A*); the latter appears to show higher than WT degradation, but most of this apparent effect is due to its overall lower inducible expression (see quantitation in Fig. 2*B*). Fig. 2 demonstrates that only the C34S mutant was unaffected by CD40 stimulation. As none of the three lysines in the RING domain prevented TRAF2 degradation, it is unlikely that ubiquitination is targeting these residues. Mutation of Cys-34 to serine, however, led to a marked block in TRAF2 degradation in response to CD40 stimulation (Fig. 2), indicating that the RING structure is required. All three of the FLAG-tagged mutants and WT mTRAF2 were recruited into membrane raft fractions after CD40 stimulation (data not shown).

CD40-induced TRAF2 Degradation Is Associated with Ubiquitination—Ubiquitination is a multistep process requiring the sequential activity of three classes of enzymes (24): ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2s), and ubiquitin protein ligases (E3s). The process of ubiquitination is initiated by the formation of a high energy thiol ester linkage between E1 and ubiquitin, which is then transferred to an E2 enzyme by the formation of another thiol ester linkage. The formation of an isopeptide bond between the substrate protein and ubiquitin is subsequently catalyzed by an E3 enzyme that also promotes the further addition of ubiquitin moieties. The net effect of these reactions is to catalyze the addition of a polyubiquitin chain to the protein substrate targeted for degradation by the 26 S proteasome. Polyubiquitinated protein is visualized on Western blot as a smear of high molecular weight species (12). Previously, we demonstrated that TRAF2 is recruited to cholesterol-rich membrane rafts following CD40 ligation and that raft-localized TRAF2 is modified by a higher molecular weight smear above the main protein band (15). However, commercially available ubiquitin-specific Abs usually fail to detect proteins modified with one or a small number of ubiquitin residues, although such modification has also been shown to regulate protein trafficking (25). Thus, to allow detection of all levels of ubiquitin modification, we used a subclone of M12.4.1 stably expressing HA epitope-tagged ubiquitin, together with inducibly expressed FLAG-mTRAF2. Fig. 3*A* demonstrates induction of TRAF2 expression in this subclone. Fig. 3*B* is an anti-FLAG blot of TRAF2 that was precipitated with anti-HA Ab and thus has one or more ubiquitin residues attached. It can be seen that both monoubiquitinated and polyubiquitinated forms of TRAF2 were significantly increased in cells stimulated through CD40. Re-probing of this blot with anti-HA (Fig. 3*C*) shows that total amounts of ubiquitinated (HA-tagged) proteins did not differ between immunoprecipitates from stimulated *versus* unstimulated cells.

TRAF2 Degradation Is Dependent upon Ubiquitination—The results in Fig. 3, together with our prior finding that TRAF2 degradation depends upon the 26 S proteasome (9) indicate that TRAF2 ubiquitination follows CD40 stimulation. To determine whether CD40-induced TRAF2 degradation is ubiquitin-dependent, we stably transfected a previously characterized CHO cell line (ts-20) (26) containing a temperature-sensitive mutant of E1 ubiquitin-activating enzyme and its parent cell line (E36) with hCD40 and FLAG-tagged mTRAF2. Transfected CHO subclones were selected to express similar levels of hCD40 (not shown). As mammalian cells contain only one known E1 enzyme, disruption of the activity of the ts E1 enzyme by increasing the temperature to 42 °C inhibits the process of ubiquitination by >90% compared with WT protein-ubiquitin-conjugating capacity (27). Stimulation of both transfected E36 and ts-20 cell lines with anti-hCD40 at 37 °C promoted TRAF2 degradation (Fig. 4, *A–C*), although the extent of degradation in the epithelial cells at 37 °C (15–20%) was less than in B cells. Stimulation of transfected E36 cell lines at

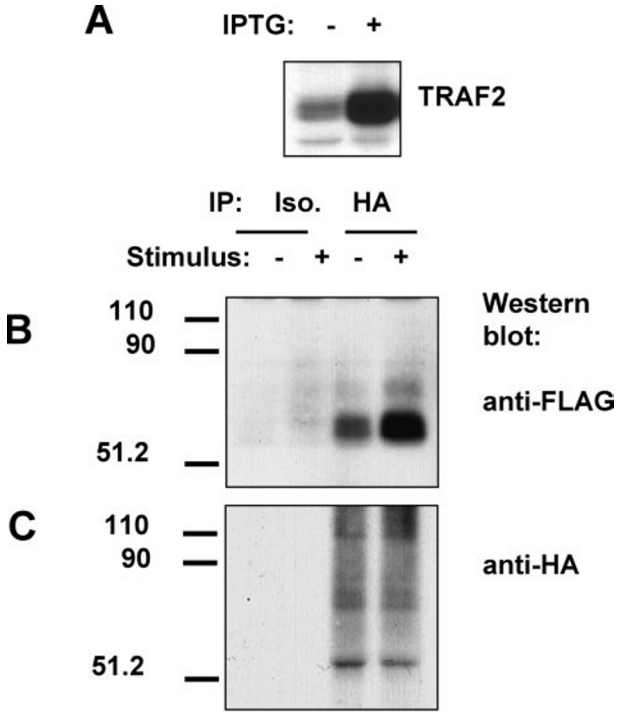

FIG. 3. **CD40-induced ubiquitination of TRAF2.** M12.4.1 cells stably transfected with HA-tagged ubiquitin and inducible FLAG-tagged mTRAF2 were incubated overnight with or without IPTG to induce TRAF2 expression and were then stimulated with either Sf9 cells (−), or Sf9 cells expressing mCD154 (+). Following stimulation, cells were lysed as described under "Experimental Procedures." Immunoprecipitations (*IP*) were performed using anti-HA or an isotype control (*iso*) Ab. *A*, anti-FLAG Western blot of cell lysates. *B*, anti-FLAG Western blots of anti-HA immunoprecipitates. *C*, blot presented in *B* was stripped and re-probed with anti-HA Ab (lane-loading control).

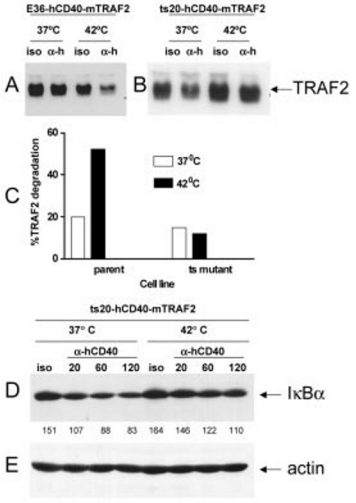

FIG. 4. **CD40-induced TRAF2 degradation requires an intact ubiquitination system.** *A* and *B,* E36 or ts-20 CHO cells expressing transfected hCD40 and TRAF2 were stimulated as described under "Experimental Procedures" with either anti-hCD40 Ab (α-*h*) or human isotype control Ab (*iso*). Following stimulation cells were lysed, and TRAF2 was immunoprecipitated with α-FLAG. Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, and blotted for TRAF2. As described in the legend to Fig. 2, total cell lysates were blotted for actin (not shown) to ensure equal amounts of protein added to precipitation beads. *C*, summary of CD40-mediated TRAF2 degradation in CHO E36 parent cells and CHO-ts20 E1 mutant cells. Values are from densitometric analysis of bands presented in *A* and represent percent TRAF2 degradation following CD40 stimulation, normalized to isotype control values. Data in *A–C* are representative of three independent experiments in both E36 and ts-20 cell lines. *D* and *E*, inhibition of CD40-induced IκBα degradation at 42 °C in temperature-sensitive E1 ligase cells. Temperature-sensitive E1 ligase cells (ts-20) stably transfected with hCD40 (5×10^6) were plated and incubated as in *A* and *B*. Cells were then stimulated with either anti-hCD40 Ab (α-*h*) for the indicated number of minutes or with isotype control Ab (*iso*) for 120 min. Total cell lysates were blotted for IκBα (*D*) or actin (*E*) as a loading control. Numbers below lanes are from densitometric analysis of IκBα bands.

42 °C increased degradation to between 40–60%, likely due to enhanced enzyme kinetics with increased temperature (Fig. 4, *A* and *C*). In marked contrast, transfected ts-20 cell lines stimulated with anti-hCD40 at 42 °C showed <15% CD40-induced TRAF2 degradation (Fig. 4, *B* and *C*). These data demonstrate dependence of CD40-induced TRAF2 degradation on ubiquitination. Degradation of IκBα, a protein known to be ubiquitinated and proteasomally degraded by NF-κB activating stimuli such as CD40, was also inhibited at 42 °C in transfected ts-20 cell lines (Fig. 4*D*).

Inhibition of TRAF2 Degradation Potentiates CD40-mediated Activation of c-Jun Kinase—Previous work in our laboratory demonstrated enhanced B cell activation in response to signaling through WT-LMP1 or a chimeric hCD40-LMP1 receptor compared with signaling through WT-hCD40 or a LMP1-hCD40 chimera (9). This enhanced signaling capacity correlates with a lack of TRAF degradation following LMP1 activation, whereas proteasome-dependent TRAF2 and TRAF3 degradation begins as early as 5 min after CD40 stimulation (9, 15). Although these correlative data are provocative, we wished to test directly whether ubiquitin-dependent TRAF2 degradation could normally limit CD40-mediated B cell activation. As TRAF2 is required for CD40-mediated JNK activation (28, 29), and the JNK pathway plays an important role in B cell activation by CD40 (30), we examined the effect of proteasome inhibition on CD40-mediated JNK activation. M12.hCD40 cells were preincubated for 2 h in the presence/absence of MG132 (a 26 S proteasomal inhibitor) before stimulation with either anti-CD40 or an isotype control Ab. Stimulation of untreated cells with anti-CD40 led to a striking (>10-fold) increase in JNK activity at 15 min, but by 30 min this activity was markedly decreased and by 60 min returned to baseline levels (Fig. 5, *A* and *B*). Stimulation of MG132-treated B cells with anti-CD40 led to a similar increase in JNK activity, but this activity was approximately twice as high as that stimulated in the absence of proteasome inhibitor. Additionally, JNK activity was sustained. At 30 min, when CD40-stimulated JNK activity is normally reduced by 3–5-fold compared with its peak at 15 min, activity was still at peak levels in MG132-treated cells. Although even in these cells, JNK activity began to decline at 60 min, the level was still similar to the peak level attained in the absence of MG132 (Fig. 5*B*). These data demonstrate that CD40-mediated JNK activation, a signal dependent upon TRAF2, is potentiated by inhibition of 26 S proteasome activity. The osmotic stressor, sorbitol, also increases B cell JNK activity in a TRAF2-independent manner (15); sorbitol-mediated activation was unaffected by MG132 treatment (not shown). This is consistent with the hypothesis that CD40-mediated JNK activation, a TRAF2-dependent pathway, is negatively regulated by ubiquitination. As shown previously (9), Fig. 5*C* demonstrates that CD40-induced TRAF2 degradation is inhibited by MG132 (as well as IκBα, a positive control). However, neither CD40 itself nor TRAF1 are degraded in response to CD40 stimulation, and there is no effect by MG132 treatment.

DISCUSSION

Considerable investigation by many laboratories has indicated that TRAF molecules play key roles in signaling by the TNF-R family, and TRAF2 associates with many members of this family. Why, then, would it be normal and desirable for

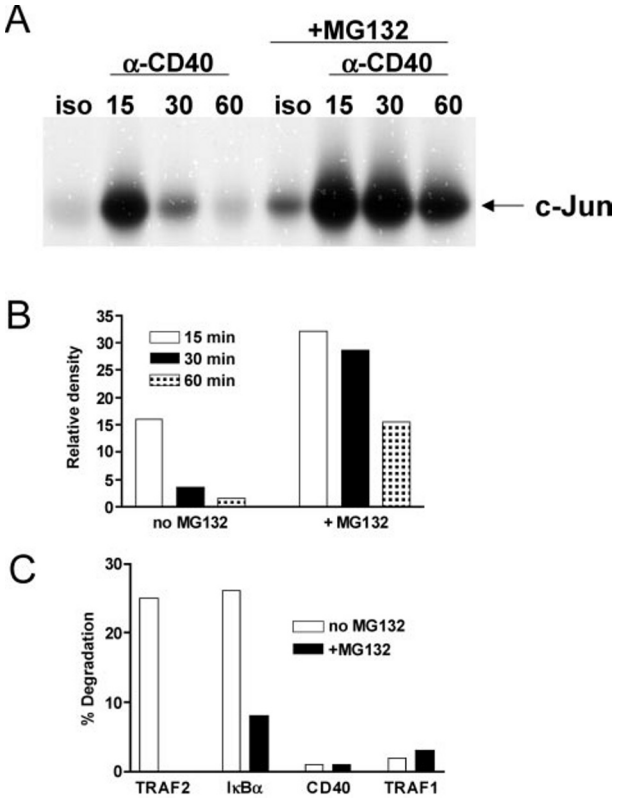

FIG. 5. **Effect of proteasomal inhibition on CD40-mediated JNK activation.** *A*, M12.4.1 B cells were preincubated in the presence/absence of MG132 for 2 h to inhibit 26 S proteasome activity and stimulated for the indicated times with either 2 μg/ml anti-CD40 or anti-CD40 isotype control Ab (*iso*). The hCD40 transfectant was used for these experiments to allow blotting for hCD40 in *C*; no mouse-specific Ab that works on Western blots is available. An *in vitro* JNK assay was performed as described under "Experimental Procedures." *B*, summary of JNK activation by CD40 with or without 26 S proteasome inhibition. Relative densitometry values of anti-CD40-stimulated samples minus values for isotype control antibody-stimulated cells are presented. Data are representative of four independent experiments. *C*, quantitation of Western blot analysis of degradation of B cell proteins in the absence (*open bars*) or presence (*filled bars*) of MG132. Cells were stimulated as above for 30 min, and whole cell lysates were subjected to SDS-PAGE and Western blotting for the indicated proteins, as described in the legend to Fig. 1. Band intensities were quantitated and normalized to actin values, as in previous figures. The 30-min time period was selected because at later time points, IκBα (37) and TRAF1 (38) are increased by CD40 stimulation.

these receptors to induce TRAF2 degradation? A number of possible explanations are worth considering. First, our recent comparison of signaling to B cells by two receptors that utilize TRAF2 indicate that a virally encoded receptor required for EBV-mediated transformation, LMP1, lacks the ability to induce degradation of TRAF2, an ability that CD40 clearly possesses (Ref. 9 and present study). This lack of TRAF degradation correlates with B cell signals from LMP1 that are amplified and sustained compared with those delivered by CD40 (9). These findings are extended in the present report by the observation that directly decreasing TRAF2 degradation using a proteasome inhibitor potentiates one of the earliest measurable events in CD40 signaling, the activation of JNK. Interestingly, the elevated and sustained JNK activation seen by CD40 in the presence of proteasome inhibition is similar to LMP1-induced JNK activation (9). Our results thus strongly suggest that receptor-mediated TRAF degradation is integral to normal feedback regulation of signaling pathways mediated by TNF-R family molecules, such as CD40. An earlier report indicating that CD30 signaling in T cells is also associated with a loss of TRAF2 (10) is consistent with this hypothesis. More recently, it was reported that TRAF6 degradation can be induced during RANKL signaling but not by receptor engagement itself; rather by ligation of the receptor for interferon-γ (31).

Many TNF-R family molecules associate with more than one type of TRAF some of which (*e.g.* TRAFs 1 and 2) can heterodimerize and some (*e.g.* TRAFs 2 and 3) that bind to overlapping regions on the receptor molecule. It is thus likely that the stoichiometry and specific composition of receptor-associated signaling complexes changes during the course of a receptor-mediated signaling cascade, and such changes may be important to the effector functions of the receptor on specific cell types. For example, our earlier studies showed that TRAF6 plays important roles in CD40-mediated B cell functions (32). However, while TRAFs 2 and 3 are rapidly modified and degraded following CD40 engagement in B cells (Refs. 9 and 15 and present study), TRAF6 does not show this CD40-mediated pattern of early modification and degradation. However, the report quoted above (31) demonstrated TRAF6 degradation in osteoclasts over a study period of up to 3 days, so it may be that both TRAF6 association and its ultimate degradation occur much later in the CD40-mediated signaling pathway in B cells. As TRAF6 binds to B cells less tightly than do TRAFs 2 and 3 (33), and requires membrane-bound CD154 rather than anti-CD40 mAb to stimulate B cell IL-6 production (32, 34) this is a reasonable possibility. Thus the nature and composition of the CD40-associated signaling complex is likely to change during the course of a CD40-induced signal cascade, and these changes may play an important role in the ability of distinct motifs in the cytoplasmic domain of CD40 to regulate different CD40 effector functions (16, 22, 34). This is likely to be the case for other TNF-R family molecules as well. In this context, the rapid triggering and subsequent degradation of some but not all TRAFs may facilitate signaling complex changes allowing for a greater number of effector functions to be regulated by an individual receptor. Additionally, some TRAFs may contribute more to early, and others to sustained, signaling induced by ligation of particular receptors. Thus, while CD40-mediated B cell activation could conceivably occur by bystander, non-cognate interactions with activated T cells, the higher avidity cognate interactions will lead to better and more sustained CD40 signaling (17), to which TRAF6 may make a larger contribution.

The present report shows that the RING structure of TRAF2 is critical in mediating its degradation. TRAF1 does not directly bind CD40 but can associate with the receptor through heterodimerization with TRAF2 (33). Thus, TRAF1 could also be recruited to membrane microdomains following CD40 engagement. We have confirmed that this occurs, but no degradation of TRAF1 is subsequently seen.[3] TRAF1 is the only TRAF molecule lacking a RING domain (35), which could explain the lack of TRAF1 degradation. Our data thus suggest that both tight association with a receptor as well as the presence of a RING domain are needed for self-initiated TRAF degradation. Future experiments will further probe how receptor engagement triggers TRAF degradation.

Our previous work comparing the signaling activities of CD40 and LMP1 as well as current work indicating enhanced and sustained CD40-mediated JNK activity in proteasome-inhibited cells suggests that this degradation could have major implications for receptor activity. It should prove of great interest to determine which other TNF family members regulate their signaling in a similar manner, as many may be regulated

[3] G. A. Bishop, unpublished data.

by TRAF degradation as well (10, 36). Exploitation of this mode of regulation in these signaling pathways may ultimately prove advantageous in modifying immune responses.

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