Members of the tumor necrosis factor receptor (TNFR) family play a variety of roles in the regulation of lymphocyte activation. An important TNFR family member for B cell activation is CD40. CD40 signals stimulate B cell TNF-α secretion, which subsequently signals via TNFR2 (CD120b) to enhance B cell activation. Although the function of the pro-apoptotic and pro-inflammatory receptor TNFR1 (CD120a) has been the subject of much research, less is understood about the distinct contributions of CD120b to cell activation and how it stimulates downstream events. Members of the tumor necrosis factor receptor family bind various members of the cytoplasmic adapter protein family, the tumor necrosis factor receptor-associated factors (TRAFs), during signaling. Both CD40 and CD120b bind TNF receptor-associated factor 2 (TRAF2) upon ligand stimulation. Wild type and TRAF2-deficient B cells expressing CD40 or the hybrid molecule (human) CD40 (mouse)-CD120b were examined. CD40- and CD120b-mediated IgM secretion were partly TRAF2-dependent, but only CD40 required TRAF2 for c-Jun N-terminal kinase activation. CD40 and CD120b used primarily divergent mechanisms to activate NF-κB, exemplifying how TNFR family members can use diverse mechanisms to mediate similar downstream events.

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Members of the TNFR family are necessary for proper activation and regulation of the immune system (1). However, signals through these receptors also contribute to various cancers and autoimmune diseases (2, 3). CD40, a 50-kDa transmembrane molecule, is expressed constitutively on B lymphocytes, dendritic cells, and monocytes/macrophages (4, 5) and is necessary for optimal antigen-presenting cell activation and function during adaptive immune responses (6). Its ligand CD154 (CD40L) is expressed on activated CD4+ T helper cells (Th) and some other cell types (7). Engagement of CD40 by CD154 leads to B cell proliferation, immunoglobulin (Ig) secretion, and isotype switching (4). In addition to its direct role in B cell activation, CD40 signaling also induces up-regulation of costimulatory and adhesion molecules as well as inflammatory lymphokines, including interleukin 6 and TNF-α. Our laboratory has previously shown that CD40-mediated Ig secretion is partially TNF-α-dependent, and the TNFR involved on B lymphocytes is CD120b (8).

The fundamental role of CD40 signals in normal immunity and pathological processes has prompted evaluation of CD40-mediated signaling cascades. We and others have shown that in B lymphocytes, CD40 can directly bind to TRAFs 2, 3, and 6, whereas TRAF1 binds CD40 primarily via heterodimerization with TRAF2 (4), although there is a small amount of direct binding (9). Binding of CD40 by CD154 causes trimerization of CD40, which allows the recruitment of TRAFs to transduce signals to downstream molecules, including NFκB (10) and members of the mitogen-activated protein kinase and stress-activated protein kinase families (11, 12). CD120b also binds TRAFs, although to what extent is relatively unknown, with the exception of TRAF2 (8), since most of these studies have been done in yeast two-hybrid and overexpression systems (13, 14). There is also conflicting evidence as to which signaling cascades are utilized by CD120b (14, 15).

Because TNF-α contributes to CD40-mediated IgM production (8), we compared the ability of CD40 and CD120b to activate c-Jun N-terminal kinase (JNK) and NFκB and investigated the role TRAF2 plays in these signaling events using previously constructed TRAF2-deficient B cell lines. CD40 required TRAF2 to activate JNK. CD120b activated JNK to a lesser degree, and this pathway was TRAF2-independent. Both CD40 and CD120b activated NFκB transcription in a partially TRAF2-dependent manner; CD120b did so exclusively via the alternate NFκB pathway, in which the p100 precursor is processed to transcriptionally active p52. CD40 was able to activate NFκB using both the classical and alternate pathways. Differences in signaling between CD40 and CD120b were associated with different kinetic profiles of TRAF2 receptor association and degradation.

**Experimental Procedures**

**Cells**

The mouse B cell lines M12.4.1 and CH12.LX as well as their stable transfectants expressing hCD40 or hCD40-hCD120b molecules have been previously described (8, 16, 17). TRAF2 was deleted from the CH12.LX cell line (CH12.T2−/−) by homologous recombination, as previously described (10). All mouse cells were maintained in RPMI 1640 containing 10% fetal calf serum (Hyclone, Logan, UT), 10 μM β-mercaptoethanol, and antibiotics (B cell medium (BCM)). Hi5 insect cells and Hi5 cells expressing mCD154 have been previously described (18, 19). These cells grow at 26 °C and rapidly die to form membrane fragments at 37 °C and, therefore, do not overgrow B cell cultures.

53222 This paper is available on line at http://www.jbc.org
Monoclonal antibodies were purified in our laboratory from hybridoma culture supernatants. The 1C10 hybridoma (anti-mCD40, rat IgG2a) (20) was provided by Dr. Frances Lund, Trudeau Institute (Saranac Lake, NY); the monoclonal antibody 72-2 (rat IgG2a isotype control) (21), MOPC-31c (mouse IgG1 isotype control), and the G28-5 (anti-CD40 mouse IgG1) hybridomas were from the ATCC (Manassas, VA). Polyclonal rabbit anti-p52, anti-p100, anti-RelB, anti-JNK (p46/54), and anti-TRAF3 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal rabbit anti-38, anti-phosphorylated IκBα and anti-phosphorylated stress-activated protein kinase/JNK were from Cell Signaling Technology (Beverly, MA). Mouse anti-actin antibody (C4) was from Chemicon International (Temecula, CA). Peroxidase-labeled goat anti-rabbit and goat anti-mouse IgG antibodies were from Bio-Rad. Recombinant mouse (rm) TNF-α was purchased from Peprotech (Rocky Hill, NJ). Enbrel (CD120b-Fc fusion protein; Immunex Corp., Seattle, WA) was obtained from the University of Iowa Hospital Pharmacy, Iowa City, IA.

IgM Secretion Assay

IgM secretion by CH12.LX or CH12.T2 cells stably transfected with hCD40 or hCD40-CD120b was measured by hemolytic plaque assay, as previously described (22). Briefly, cells were cultured for 72 h with various stimuli, then the viable cells were counted by ethidium bromide-acridine orange staining (23). The B cells were mixed with sheep red blood cells and guinea pig complement, transferred to chamber slides, and incubated for 30 min at 37 °C. During this incubation, IgM secreted by activated B cells bound to surrounding sheep red blood cells (expressing the antigen for which CH12.LX IgM is specific, phosphatidylcholine (24)) and fixed complement, causing the red cells to lyse. Zones of clearing or “plaques” surrounding the activated B cells were counted using a low power microscope. Results are presented as the ratio of plaque-forming cells to viable cells. As previously described (22, 25), CH12.LX does not express detectable levels of surface CD32, and thus, intact mouse IgG antibodies do not have CD32-specific effects on these cells.

NfκB Dual Luciferase Reporter Assay

CH12.LX or CH12.T2 cells (1.5 × 10^6) stably transfected with hCD40 or hCD40-CD120b were transiently transfected with 20 ng of 4× NFκB luciferase reporter plasmid (26) and 1 ng of Renilla luciferase vector (pRL-null; Promega, Madison, WI) by electroporation. Cells were incubated on ice for 15 min, then stimulated (2 × 10^6 cells/ml) for 6 h with 100 ng/ml recombinant TNF-α or 10 ng/ml anti-mCD40, anti-hCD40, or isotype control antibodies. Alternatively, cells were co-cultured with Hi5 insect cells (19) (1:4 Hi5:B cells) infected with baculovirus expressing either mouse or human CD154. In some experiments, cells were rested for 30 min at 7°C with 10 ng/ml rmTNF-α (MP6-XT3 + MP6-XT22), Enbrel, or isotype control antibodies. After stimulation, cells were pelleted, lysed, and assayed for relative luciferase activity (NFκB:Renilla) per the manufacturer’s protocol (Promega) using a Turner BioSystems (Sunnyvale, CA) 20/20 luminometer, with settings of 2-s delays followed by a 10-s read.

TRAF Degradation/Isol β/JNK Assays

M12.4.1 cells (2 × 10^6), CH12.LX, or CH12.T2 cells stably transfected with hCD40 or hCD40-CD120b were stimulated in 1 ml of culture medium for various time points (37°C) with 5 μg of IκBα, TRAF, or 10 μg (JNK) of anti-hCD40 or Hi5 insect cells expressing CD154 (1:4 ratio with B cells, IκBα) to induce degradation (TRAF) or phosphorylation/degradation (IκB/β/JNK) of the proteins blotted. The cells were pelleted by centrifugation, lysed, and analyzed by SDS-PAGE and Western blotting. Peroxidase-labeled antibodies were visualized on Western blots using a chemiluminescent detection reagent (Pierce). Chemiluminescence was quantified using a FujiFilm LAS-1000 imaging system (FujiFilm Medical Systems, Ltd., Stamford, CT).

NFκB2 Activation

Total Cell Lysate Analysis—M12.4.1 cells (2 × 10^6) stably transfected with hCD40 or hCD40-CD120b were stimulated in 1 ml of culture medium for various time points (37°C) with 5 μg of anti-hCD40 (or isotype control) to induce processing of p100 to p52 and RelB activation. The cells were pelleted by centrifugation, lysed, and analyzed by SDS-PAGE and Western blotting. In some experiments cells were preincubated for 30 min at 37°C with 10 μg/ml anti-TNF-α (MP6-XT3 + MP6-XT22).

Nuclear Translocation of p52 and RelB—CH12.LX or CH12.T2 cells stably transfected with hCD40 or hCD40-CD120b were stimulated in 1 ml of culture medium for various time points (37°C) with 10 μg of anti-hCD40 (or isotype control). Cellular and nuclear fractions were prepared as described previously (27). Briefly, cells were lysed with sucrose buffer (320 mM sucrose, 3 mM CaCl₂, 2 mM magnesium acetate, 0.1 mM EDTA, 10 mM Tris-HCl, pH 8, and 0.4% Nonidet P-40) and 0.1% sodium dodecyl sulfate. Nuclei were then washed once with sucrose buffer without Nonidet P-40 and resuspended in 30 μl of low salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM MgCl₂, 20 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride). High salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM MgCl₂, 300 mM NaCl, 0.15 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) was added slowly in two 15-μl additions while mixing. Nuclei were incubated on a rotator for 30–45 min at 4°C. Protease and phosphatase inhibitors were added to the cellular and nuclear fractions, which were then stored at −20°C.

JNK Activation Assay

CH12.LX or CH12.T2 cells stably transfected with hCD40 or hCD40-CD120b (2 × 10^6) were rested for 30 min, then stimulated in 1 ml of culture medium for 5 min (37°C) with 10 μg of anti-mCD40 or anti-hCD40 (or respective isotype controls). Cell lysates were prepared, and JNK activity was measured using an in vitro kinase assay, as previously described (28). Reactions were separated by SDS-PAGE, and phosphorylated c-Jun was visualized by autoradiography of dried gels. Radiolabeled kinase substrate was quantified with the Packard Instant Imager (Packard Instrument Co.).
TRAF Recruitment to Receptors in Detergent-insoluble Fractions

M12.4.1 cells (1 × 10⁷) stably transfected with hCD40 or hCD40-CD120b were stimulated for 5 min with anti-mCD40 (versus isotype control), mIC or anti-hCD40 (versus isotype control, hIC) Ab or with Hi5 insect cells transfected with WT baculovirus (CHI) or recombinant baculovirus expressing mCD154 or hCD154. Cell lysates were assayed for kinase activity by phosphorylation of a glutathione S-transferase-c-Jun substrate as shown by autoradiography. Band densities (cpm) were quantified with the Packard Instant Imager. B, alternatively, after stimulation for 5–60 min, cells were lysed as under “Experimental Procedures,” and lysates were analyzed by SDS-PAGE and Western blot for phosphorylated (upper panel) or total (lower panel) JNK. Similar results were obtained in two independent experiments. m, mouse.

RESULTS

To study potential differences in signaling by the cytoplasmic domains of CD40 and CD120b, we previously stably transfected mouse B cells lines M12.4.1, CH12.LX, and CH12.T2⁻/⁻ with a construct encoding extracellular and transmembrane human CD40 domains fused to the C-terminal cytoplasmic mouse CD120b domain (hCD40-CD120b) (8). Alternatively, cells were stably transfected with a construct encoding hCD40. The cytoplasmic domains of mouse and hCD40 are similar in amino acid sequence and indistinguishable in function (30), allowing use of endogenous mCD40 in the B cell lines as an important control. Use of the hybrid hCD40-CD120b molecule avoids the problem of lack of available reagents that efficiently detect or immunoprecipitate endogenous CD120b from mouse cells. Neither normal mouse B cells nor B cell lines express detectable CD120a mRNA or protein, and the hCD40-CD120b molecule mimics closely the effects of exogenous TNF-α on B cells (8). TNF-α was used as a control throughout the study to validate hybrid molecule findings with endogenous CD120b, as technically feasible.

The Role of TRAF2 in CD40- and CD120b-mediated IgM Secretion—TNF-α promotes antibody production in vitro and in vivo (31). We have previously shown that TNF-α induced via CD40 stimulation contributes to CD40-mediated antibody production in B cells via binding to CD120b (8). Both CD40 and CD120b bind TRAF2. Inducible expression of a truncated, dominant negative DN-TRAF2 inhibits CD40 mediated Ig production (18). However, we wanted to examine receptor signaling in the complete absence of TRAF2, avoiding the complications of variable DN-TRAF2 expression and the severe viability and developmental defects of TRAF2⁻/⁻ mice (32). To this end, hCD40 or hCD40-CD120b was stably transfected into TRAF2-deficient CH12.T2⁻/⁻ cells (10), and activation was compared with similarly transfected wild type (WT) CH12.LX cells. Stimulation CH12.LX cells through endogenous mCD40 or the
transfected hCD40 molecule resulted in an increase in IgM-secreting B cells. The activation of TRAF2-deficient cells by either stimulus was diminished (Fig. 1). This was true whether stimulating via anti-CD40 Ab (Fig. 1A) or insect cells expressing CD154 (Fig. 1B). Stimulating via the hCD40-CD120b hybrid molecule or using rmTNF-α/H9251 to activate endogenous CD120b induced IgM-secreting cells to a lesser extent than CD40, as previously reported (8). This effect was also diminished in the absence of TRAF2.

Differential Role of TRAF2 in CD40- and CD120b-mediated JNK Activation—CD40-mediated JNK activation contributes to downstream antibody secretion, and we have previously shown that TRAF2 plays a role in this process (10). Results shown in Fig. 1 suggest that TRAF2 may also be required for CD40 and CD120b-mediated JNK activation. We examined the ability of hCD40 or hCD40-CD120b to stimulate B cell JNK activation in the presence or absence of TRAF2 using an in vitro kinase assay (Fig. 2A). Stimulating via hCD40-CD120b induced less phosphorylation of the glutathione S-transferase-c-Jun substrate than stimulating B cells via mouse or human CD40 whether using Ab or CD154 as the stimulus. Unlike CD40-induced JNK activation, which was dramatically diminished in CH12.T2−/− cells, no decrease in hCD40-CD120b-induced JNK activation was seen in the absence of TRAF2. We predicted that this result might reflect the phosphorylation state of JNK after hCD40 or hCD40-CD120b activation in the presence or absence of TRAF2. Fig. 2B demonstrates that hCD40-CD120b-mediated activation led to less phosphorylation of JNK than hCD40-mediated activation. However, whereas hCD40-mediated JNK phosphorylation was diminished in CH12.T2−/− cells, hCD40-CD120b-mediated JNK phosphorylation was actually enhanced in the TRAF2-deficient cells.

Roles of TNF-α and TRAF2 in hCD40-mediated NFκB Activation—CD40-mediated NFκB activation contributes to immunoglobulin secretion (33), but the relative role of TNF-α in this process is unknown. It is also unclear to what extent CD120b can induce NFκB activation in B cells given the mixed results...
produced in overexpression systems (34, 35). To address this question, M12.4.1 cells stably transfected with hCD40 or hCD40-CD120b were evaluated for their ability to activate an NFκB-driven luciferase reporter gene upon receptor engagement (Fig. 3A, top panel). Both CD40 (mouse or human) and hCD40-CD120b were able to activate NFκB-mediated transcription. The CD120b response was approximately one-third that of the CD40 response. Blocking TNF-α binding with antagonistic antibodies or Enbrel, a CD120b-Fc fusion protein, before receptor activation caused a 30% decline in hCD40-mediated NFκB activation, demonstrating a possible role for TNF-α in CD40-mediated NFκB activation. As an additional control there was no detectable affect of TNF-α blockade on hCD40-CD120b-mediated NFκB activation (Fig. 3A, bottom panel).

To compare the role of TRAF2 in hCD40 versus hCD40-CD120b-mediated NFκB activation, we examined CH12.LX- and TRAF2-deficient CH12.T2 cells (Fig. 3B). As in the M12.4.1 cells, both CD40 (mouse and human) and CD120b (via the chimeric receptor or endogenous CD120b stimulated with rmTNF-α) were able to activate NFκB, but CD120b did so to a lesser extent. This was true whether stimulating with anti-mCD40 Ab (Fig. 3B, upper panel), rmTNF-α, or CD154 (Fig. 3B, lower panel). CH12.T2−/− cells showed diminished NFκB activation after CD40 or CD120b stimulation, but activity was not completely abolished. This is in agreement with previous findings that CD40 mutants defective in TRAF2 binding are still able to activate NFκB (36).

Mechanisms of NFκB Activation in B Cells by CD40 Versus CD120b—Early events in NFκB activation have been shown to include phosphorylation and degradation of IκB (37). However, recent studies suggest that an alternate pathway for activating NFκB, in which p100 is processed to p52 and shuttled to the nucleus by RelB, is also used by some TNF receptor family members, including CD40 (38), lymphotoxin β receptor (39), and the B cell activating factor of the TNF family receptor (40). To test which NFκB activation pathways are utilized by CD40 and CD120b in B cells, M12.4.1 cells were activated via CD40 or hCD40-CD120b and assayed for IκB phosphorylation and degradation (classical pathway, Fig. 4) or processing of

![Phosphorylation and degradation of IκBα by hCD40 versus hCD40-CD120b](image-url)
p100 to p52 (alternate pathway, Fig. 5). Both mouse and human CD40 were able to rapidly stimulate phosphorylation and degradation of IκBα, but hCD40-CD120b did not, either with a stimulus of agonistic anti-CD40 antibody (Fig. 4, A–B) or CD154 (Fig. 4, C–D). However, both hCD40 and hCD40-CD120b were able to activate the alternate pathway (Fig. 5), and CD120b was more efficient at inducing p100 processing to p52 and RelB activation. Furthermore, CD40-mediated activation of the alternate NFκB pathway was TNF-α dependent, as blocking TNF-α binding before CD40 activation greatly diminished activation of the alternate pathway (Fig. 5).

To confirm findings in Fig. 5, we examined activation of the alternate pathway by evaluating cytoplasmic and nuclear fractions post-stimulation for p52 and RelB activation in the cytoplasm and movement to the nucleus. To determine the role of TRAF2 in this process, transfected CH12.LX or CH12.T2 cells were stimulated via hCD40 or hCD40-CD120b and assayed for p100, p52, and RelB levels (Fig. 6). As in the M12.4.1 cells (Fig. 5), hCD40-CD120b in CH12.LX cells was able to activate the alternate pathway more efficiently than hCD40, with a greater diminution of p100, concomitant increase of p52, and accumulation/degradation of RelB in the cytoplasm (Fig. 6, A–B). This was followed by a greater increase of p52 and RelB levels in the nucleus (Fig. 6, C–D). Activation of the alternate pathway by hCD40 or hCD40-CD120b in TRAF2-deficient cells was appreciably decreased to base-line levels in the cytoplasmic fraction (Fig. 6, A–B) but not completely diminished in the nucleus, especially with hCD40-CD120b stimulation (Fig. 6, C–D). Some TRAF2-independent activation may, thus, occur.

Differential Kinetics of TRAF2 and TRAF3 Association and Degradation Induced via CD40 Versus CD120b—Association of TRAFs with CD40 within lipid membrane rafts has been shown to be vital for downstream signaling events, including JNK and NFκB activation (41, 42). A further level of signaling regulation may occur via TRAF degradation after receptor association (29, 41, 43). We hypothesized that differences in signaling mechanisms between CD40 and CD120b might also be manifest in the association of TRAFs with CD40 versus CD120b. Because TRAF2 and TRAF3 have been shown to act as positive and negative regulators of CD40 signaling, respectively (16), we focused upon these molecules. We examined movement to rafts and receptor association of TRAF2 and TRAF3 in Brij soluble (non-raft) and insoluble (lipid raft) fractions after hCD40 or hCD40-CD120b engagement (Fig. 7) in M12.4.1 cells. We found that both TRAF2 and TRAF3 association with CD40 was strongest within 15 min of agonistic antibody stimulation and then gradually declined over the 1-h time period, as demonstrated by both raft movement (Fig. 7, A and B, top panel of each) and CD40 binding (Fig. 7, A and B, lower panel of each). However, association of TRAF2 and TRAF3 with the CD120b cytoplasmic tail was slower. Similar results were seen stimulating with CD154 and in CH12.LX cells (data not shown). Furthermore, there was no decrease of TRAF2 or TRAF3 association with the CD120b cytoplasmic tail over the 1-h time period. This prompted us to evaluate TRAF degradation after stimulation via hCD40 or hCD40-CD120b (Fig. 8). As seen previously (10, 29, 41), whether stimulating mouse or human CD40, both TRAF2 and TRAF3 are consistently degraded over the 2-h (similar results seen stimulating with CD154 and in CH12.LX cells, data not shown). However, hCD40-CD120b-mediated activation induced less efficient TRAF2 degradation and very little TRAF3 degradation. This was unchanged up to 6 h post-stimulation (data not shown).

**DISCUSSION**

The cytoplasmic tails of both CD40 and CD120b bind TRAF2, suggesting overlapping roles for these two receptors in B cell activation. Data presented here show that both receptors were
able to stimulate IgM secretion (Fig. 1) and the activation of JNK (Fig. 2) and NFκB (Fig. 3). However, CD120b was less efficient at promoting these events than CD40 and used the alternate pathway of NFκB activation (Figs. 5–6) instead of the classical pathway primarily used by CD40 (Fig. 4).

Both CD40- and CD120b-mediated IgM production are partially dependent on TRAF2, as seen in our TRAF2-deficient cells (Fig. 1) and in previous work with dominant negative TRAF2 molecules (8). We predicted that this would also be true for signals leading to IgM production, including JNK and NFκB. However, in the case of JNK activation, CD40 was dependent on TRAF2, but CD120b was not (Fig. 2). CD120b-mediated JNK activation in B cells may be TRAF2-independent. CD120b-mediated JNK phosphorylation and in vitro kinase activity actually improved in the absence of TRAF2 (Fig. 2), suggesting a possible negative role for TRAF2 in this aspect of CD120b signaling. Varied use of TRAFs among TNF receptors is not unusual. In CD40-mediated signaling, TRAF2 plays a positive role, whereas TRAF3 is a negative regulator (16). However, in the case of the CD40 viral mimic LMP1, encoded by the Epstein-Barr virus, TRAF3 positively regulates signaling (44). We find here that CD120b was able to recruit and bind

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**Fig. 6. Role of TRAF2 in CD40- versus CD40-CD120b-mediated nuclear translocation of p52 and RelB.** WT CH12.LX or TRAF2 

CH12.T2-/- cells transfected with hCD40 or hCD40-CD120b were stimulated with anti-hCD40 or isotype control (IC) Ab for 5 min to 6 h. Cytoplasmic (A–B) and nuclear (C–D) fractions were isolated as described under “Experimental Procedures” and analyzed by SDS-PAGE and Western blot for p100/p52 or RelB. Blots were stripped and reprobed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a lane-loading control. B and D, density of bands in A and C as a proportion of BCM-stimulated cells normalized to the value of the GAPDH band (X, isotype control). Similar results were obtained in 3 independent experiments.
TRAF3 in membrane lipid rafts (Fig. 7), and it has been suggested that membrane localization of TRAF3 enables JNK activation (45), making this TRAF a possible candidate as a positive regulator for CD120b-mediated JNK activation.

The distinct use of the classical and alternate NFκB pathways by CD40 and CD120b illustrates how two TNFR family members can mediate similar downstream events through different mechanisms. CD120b exclusively used the alternate pathway to activate NFκB (Figs. 5–6), whereas CD40 used this pathway to a lesser extent and efficiently activated the classical pathway (Fig. 4). Although CD40 (38, 46) and its viral mimic LMP1 (47) have been shown to activate both NFκB pathways, other TNFR family members, including lympho-toxin β receptor (48) and the B cell activating factor of the TNF family receptor (40), like CD120b, almost exclusively use the alternate pathway. This may explain why we (Fig. 3) and others (35) find CD120b to be relatively inefficient at NFκB-dependent transcription compared with other TNFR family members, including CD40 and CD120a. The role of TNF-α in CD40-mediated NFκB-dependent transcription (Fig. 3A) also relies upon activation of the alternate pathway. Blocking TNF-α had no effect on CD40 driven IκBα phosphorylation and degradation (data not shown) but inhibited the ability of CD40 signals to activate the alternate pathway (Fig. 5).

Fig. 7. Association of TRAF2 and TRAF3 with hCD40 and hCD40-CD120b. A, M12.4.1 cells stably transfected with hCD40 or hCD40-CD120b were stimulated with anti-hCD40 or isotype control (IC) Ab for 15–60 min, and detergent-soluble (S lanes) and insoluble (I lanes) fractions were isolated as described under “Experimental Procedures.” Protein samples were analyzed by SDS-PAGE and Western blot (top panel) for TRAF2 and TRAF3. hCD40 was immunoprecipitated (IP) from soluble and insoluble fractions as described under “Experimental Procedures,” and the precipitated proteins were identified by SDS-PAGE and Western blot analysis (bottom panel). B, density of bands in A as a proportion of detergent insoluble:soluble fractions in raft movement (top panel) and hCD40-immunoprecipitated (bottom panel) of TRAF2 and TRAF3 in A (X, isotype control). Similar results were obtained in two additional experiments.
TRAF2 plays a direct role in p100 stabilization and RelB activation in the cytoplasm (Fig. 6, A–B), both of which play key roles in activation of the alternate pathway (49, 50). This led to a reduction in nuclear translocation of p52 and RelB (Fig. 6, C–D). However, we consistently find some receptor-mediated accumulation of p52 and RelB in the nucleus over time despite the absence of TRAF2, suggesting a TRAF2-independent mechanism. We have already shown that TRAF2 and TRAF6 have overlapping roles in CD40-mediated 1xIκB phosphorylation and degradation (10). It has also been suggested that TRAF3 plays a role in regulation of the NFκB-inducing kinase (NIK) (51), an early kinase in the alternate pathway. The TRAF binding site of LMP1, which requires TRAF3 for many activation signals (44), has been shown to contribute to the LMP1 ability to activate the alternate pathway (52).

It was somewhat unexpected to find that TRAF3 bound the cytoplasmic tail of CD120b. However, previous studies of CD120b-TRAF association have used yeast two-hybrid and overexpression systems, and we have previously found that binding of TRAFs to receptors in such systems may not reflect their binding to normal levels of receptors in B cells (53). The relatively inefficient degradation of TRAF2 and perhaps TRAF3 may explain why CD120b was more efficient at activating the alternate NFκB pathway compared with CD40. This pathway takes hours (instead of minutes) to activate and may not be as sensitive to the delayed association of TRAFs with CD120b compared with CD40.

The recent focus on TNF-α and TNFR family members as therapeutic targets for inflammatory disease, including rheumatoid arthritis (54) and heart disease (55), has prompted the investigation of unique and overlapping signaling mechanisms of TNFR family members. CD40 (56) and CD120b (57) have both been implicated in the inflammatory process, and evaluating the role of TRAF2 in CD40 and CD120b signaling has uncovered distinct mechanisms of B cell activation. CD120b activates NFκB via the alternative and not the classical pathway, making this a promising avenue for future therapy design, as the alternate pathway plays a significant role in the humoral immune response (58) and sustaining autoimmune B cells (59).

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