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Specificities of CD40 signaling: Involvement of TRAF2 in CD40-induced NF-κB activation and intercellular adhesion molecule-1 up-regulation

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ABSTRACT **Several tumor necrosis factor receptor-associated factor (TRAF) family proteins including TRAF2, TRAF3, TRAF5, and TRAF6, as well as Jak3, have been implicated as potential mediators of CD40 signaling. An extensive *in vitro* binding study indicated that TRAF2 and TRAF3 bind to the CD40 cytoplasmic tail (CD40ct) with much higher affinity than TRAF5 and TRAF6 and that TRAF2 and TRAF3 bind to different residues of the CD40ct. Using CD40 mutants incapable of binding TRAF2, TRAF3, or Jak3, we found that the TRAF2-binding site of the CD40ct is critical for NF-κB and stress-activated protein kinase activation, as well as the up-regulation of the intercellular adhesion molecule-1 (ICAM-1) gene, whereas binding of TRAF3 and Jak3 is dispensable for all of these functions. Overexpression of a dominantly active IκBα strongly inhibited CD40-induced NF-κB activation, ICAM-1 promoter activity, and cell-surface ICAM-1 up-regulation. These studies suggest a potential signal transduction pathway from the CD40 receptor to the transcriptional activation of the ICAM-1 gene.**

CD40 is a 50-kDa glycoprotein, a member of the tumor necrosis factor receptor (TNFR) superfamily, and is expressed on a variety of cells including B cells. Studies with X-linked hyper-IgM syndrome, a genetic defect in the corresponding CD40L gene that is expressed predominantly by CD4+ T helper cells, and studies with mice lacking either the CD40 or CD40L gene, have demonstrated that interaction between CD40 and CD40L during T and B cell contact is essential for all events in T cell-dependent antigen responses, including germinal center formation, Ig isotype switching, antibody affinity maturation, memory B cell formation, and T cell activation (1–4). In cell culture systems, stimulation of CD40 receptor with either anti-CD40 antibodies or CD40L rescues B cells from apoptosis, promotes B cell proliferation, and induces Ig isotype switching in the presence of cytokines (2).

The pleiotropic biological roles mediated by CD40 raise an important issue regarding how a single receptor generates intracellular signals capable of mediating such diverse biological phenomena. Studies using yeast two-hybrid screening and *in vitro* binding assays have led to the identification of TNFR-associated factor (TRAF) family proteins 2, 3, 5, and 6 as proteins associated with CD40 (5–9). In addition, Janus kinase 3 (Jak3) also has been reported recently to associate with the CD40 cytoplasmic tail (CD40ct) (10). The TRAF family consists of six known family members, TRAFs 1–6 (1, 5–9, 11–15), which are capable of interacting with various TNF and non-TNF receptors. Overexpression of TRAFs 2, 5, and 6, but not TRAFs 1, 3, and 4, in transient transfection experiments activates both the NF-κB and stress-activated protein kinase (SAPK) signal transduction pathways (7, 8, 11, 13, 16, 17).

To better understand the contributions of the different CD40ct-interacting adapter molecules to specific CD40 signaling pathways and CD40-mediated biology, we initiated our studies by examining the relative binding affinities of TRAFs 2, 3, 5, and 6 and Jak3 with the CD40ct. It was found that the binding affinities of TRAFs 2 and 3 for the CD40ct are much greater than those of TRAFs 5 and 6. Using human CD40 mutant constructs that specifically abolish binding of TRAF2, TRAF3, or Jak3 to the CD40ct, we also found that the TRAF2-binding site in the CD40ct is required for CD40-mediated NF-κB and SAPK activation. Finally, we demonstrate that CD40-mediated up-regulation of ICAM-1, a cell-adhesion molecule important for cell–cell interactions and inflammation, is mediated through NF-κB signaling by TRAF2.

MATERIALS AND METHODS

Plasmids and Expression Vectors. Full-length wild-type human CD40 and its mutant derivatives were generated with PCR end primers and internal primers with appropriate mutations and cloned into the *Bam*HI and *Sal*I sites of the pBABEpuro vector (18, 19). Glutathione *S*-transferase (GST) fusion proteins all were constructed in the pGex2T prokaryotic gene fusion expression vector (Pharmacia). The construction of GST-C17, C10N, C10C, and the full-length GST-mCD40ct has been described previously (19). Constructs used for the alanine-scanning mutagenesis were cloned similarly. Expression was confirmed by Coomassie staining. TRAF constructs were generated by PCR and cloned into the *Bam*HI and *Not*I sites of the PEBB-Flu-tagged mammalian expression vector. The TRAF2 and TRAF3 templates were derived from constructs used previously (19). pME-TRAF5 template used for PCR was a gift from Jun-ichiro Inoue (University of Tokyo). The TRAF6 PCR product, generated from a cDNA library, was digested with *Bgl*II and *Not*I and cloned into the *Bam*HI and *Not*I sites of the PEBB-Flu vector. The pGL 1.3, pGL 1.3κB-, and the $(\kappa B)_3$-IFN-LUC luciferase reporters have been described previously (20, 21). The pBABEpuro CD40L construct has also been described previously (19).

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Abbreviations: TRAF, tumor necrosis factor receptor-associated factor; Jak, Janus kinase; CD40ct, CD40 cytoplasmic tail; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase; ICAM-1, intercellular adhesion molecule 1; IκB-α, inhibitor of NF-κBα; GST, glutathione *S*-transferase; CD40L, CD40 ligand (gp39).

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Cell Culture and Transfection. 293T cells and BOSC packaging cells were maintained in DMEM (GIBCO/BRL) supplemented with 10% fetal bovine serum (FBS) and were transfected by standard calcium phosphate methods. A549 human lung carcinoma cells, WEHI 231 murine B cell lymphoma cells, BI-141 murine T cell hybridoma cell lines, and Daudi human Burkitt's lymphoma B cell lines were maintained in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin. Transient transfections with reporter constructs were performed with the Superfect Transfection Reagent (Qiagen, Chatsworth, CA). Harvested samples were assessed for luciferase activity by measurement with Promega luminol substrate. Infections of the BI-141 and WEHI 231 cells were performed by the use of BOSC packaging cells as described previously (18, 22). For electroporation, Daudi cells were electroporated at 250 V/975 μF and selected with 1 mg/ml of active G418 (Mediatech, Washington, DC).

***In Vitro* Binding Assays, Electrophoretic Mobility-Shift Assays, and *in Vitro* Kinase SAPK Assays.** GST pull-down assays were performed as described previously (19). For gel-shift and SAPK assays, cells were treated as indicated in the text. Nuclear extracts were prepared and used in binding reactions with a γ-P^{32}-labeled major histocompatibility complex II NF-κB specific hairpin oligonucleotide (20) using previously published protocols (23). Endogenous SAPK activity was assessed by immunoprecipitation with anti-c-Jun N-terminal kinase 1(JNK1) polyclonal antibodies (Santa Cruz Biotechnology) using previously published protocols (24).

Fluorescence-Activated Cell Sorter (FACS) Analysis. To assess expression of the human CD40 constructs, BI-141 infectants and parental cells were stained with fluorescein isothiocyanate (FITC)-conjugated murine anti-human CD40 mAb (clone 5C3; PharMingen) and analyzed by FACScan flow cytometer. To assess ICAM-1 induction, WEHI231 or Daudi cells were stimulated for 24 hr with 1 μg/ml of G28.5 or with soluble CD40L. Samples then were stained with *R*-phycoerythrin-conjugated anti-murine ICAM-1 antibodies (clone 3E2; PharMingen) or FITC-labeled anti-human ICAM-1 antibodies (PharMingen) and analyzed by FACScan flow cytometer.

RESULTS

TRAF2 and TRAF3 Bind to the CD40ct with Greater Affinity than TRAF5 and TRAF6. TRAF2, TRAF3, TRAF5, and TRAF6 have been shown to bind CD40, based on yeast two-hybrid assays or *in vitro* pull-down assays (5–9). To determine the relative affinities of the TRAF proteins for the CD40ct, the full-length cDNAs of TRAFs 2, 3, 5, and 6 were subcloned into the pEBB-Flu vector, which allows for equivalent detectability with antibodies against the Flu-epitope. When 293T cell extracts overexpressing similar amounts of TRAF proteins were incubated with GST-tagged human CD40ct bound to glutathione beads, strong associations were detected with the CD40ct and TRAFs 2 and 3 whereas the interactions with TRAF 5 and, especially, TRAF6 were considerably weaker (Fig. 1*A*).

Experiments similar to those described above were carried out to test the association of Jak3 with the CD40ct, which had been reported previously (10). Despite the use of a high-expression vector, pEBB-Flu-Jak3, we were unable to detect the precipitation of Jak3 with GST-CD40ct (data not shown).

TRAF2 and TRAF3 Bind Overlapping but Distinct Motifs in the CD40ct. Because of the very weak interactions of TRAF5 and TRAF6 with the CD40ct and the problems posed by our ability to detect them, we focused our subsequent efforts on mapping the binding sites for only TRAF2 and TRAF3 on the CD40ct with the aim of identifying CD40ct mutants that could specifically bind either TRAF2 or TRAF3. Using GST pull-down assays, we previously had identified a 17-aa fragment

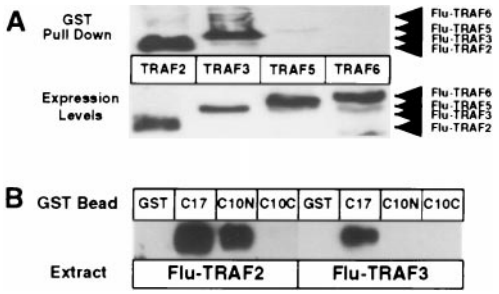

FIG. 1. Characterization of the relative binding affinities of TRAFs 2, 3, 5, and 6 for the CD40 cytoplasmic tail and determination of the minimal binding regions within the CD40 C17 region. (*A*) TRAFs 2 and 3 bind to the CD40ct with much greater affinity than TRAFs 5 and 6. The same amount of glutathione beads bound with the GST-mouse CD40ct fusion protein was incubated with extracts from 293T cells transfected with pEBB-Flu TRAF2, pEBB-Flu TRAF3, pEBB-Flu TRAF5, or pEBB-Flu TRAF6. The beads were washed, and bound proteins were detected by Western blot analysis with anti-Flu antibodies (Fig. 1*A Upper*). Equal amounts of extracts (5% of input used for GST pull-down) were analyzed by Western blot analysis with anti-Flu antibodies to ensure similar levels of expression of the different Flu-tagged TRAFs (Fig. 1*A Lower*). (*B*) The minimum binding regions of TRAF2 and TRAF3 within the C17 region are different. Lysates from 293T cells expressing Flu-TRAF2 or Flu-TRAF3 were incubated with GST, GST-C17, GST-C10N, or GST-C10C fusion proteins immobilized on glutathione beads. The bound proteins then were analyzed by Western blot analysis with anti-Flu antibodies.

(C17) corresponding to amino acid residues 230–246 of the CD40ct that could bind to TRAF3 as strongly as the entire CD40ct (19). This C17 peptide also was capable of binding to TRAF2 as strongly as the full-length CD40ct (Fig. 1*B*). This C17 fragment was dissected further into two overlapping regions corresponding to the first 10 aa (the C10N region) or the last 10 aa (the C10C region), and the corresponding fragments were assessed for interactions with TRAFs 2 and 3. The C10N region was capable of interacting with TRAF2 but not with TRAF3, whereas the C10C was incapable of interacting with either TRAF2 or TRAF3 (Fig. 1*B*). These results suggest that whereas the C17 region within the CD40ct is close to the minimal-binding motif for TRAF3, the minimal-binding region for TRAF2 lies within the C10N region.

To further define the binding specificities between TRAF2 and TRAF3, we carried out alanine-scanning mutagenesis to replace with alanine, one by one, each of the amino acids of the 17-aa fragment in the CD40ct. The GST fusion proteins containing wild-type C17 and its mutant variants then were used in the GST pull-down assays to determine the effects of these mutations on their interactions with TRAF2 and TRAF3. The mutations that abolished the interaction of TRAF2 with the C17 region were P230A, V231A, Q232A, E233A, and T234A (Fig. 2*A*). All fall within the C10N fragment. Mutations that abolished TRAF3 binding included T234A (which also abolished TRAF2 binding), L235A, and V241A (Fig. 2*B*). They span the junction of C10N and C10C. To determine the effects of mutations in the context of full-length CD40ct, the GST fusion constructs containing the full-length wild-type CD40ct and full-length CD40ct carrying mutations such as P207A, Q232A, T234A, and V241A were examined, and their interaction patterns with TRAF2 and TRAF3 were observed to be the same as their corresponding GST-C17 constructs (data not shown). Thus, the binding sites for TRAF2 and TRAF3 differ significantly, thereby allowing us to make mutations that can selectively interfere with the binding of these factors to CD40.

The role of the one amino acid in common for binding TRAF2 and TRAF3, T234, was studied by mutating it in the C17 fragment to either serine or valine, generating GST fusion

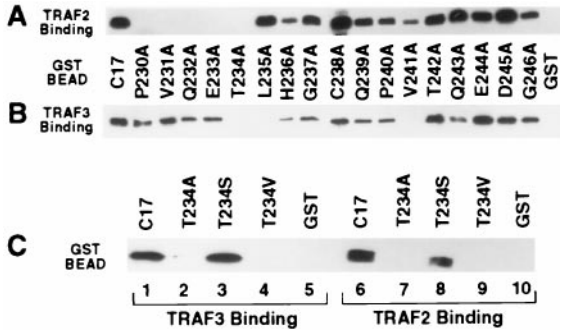

FIG. 2. Dissection of the CD40ct by alanine-scanning mutagenesis. (*A* and *B*) TRAF2 and TRAF3 contact overlapping but different sets of amino acid residues in the CD40ct. The same amount of GST protein and GST fusion proteins containing either wild-type C17 or one of 17 mutated forms of C17, with 1 aa residue replaced by alanine, was incubated with 293T cell extracts overexpressing Flu-tagged TRAF2 (*A*) or TRAF3 (*B*). The bound Flu-TRAF2 and Flu-TRAF3 were detected by Western blot analysis using anti-Flu antibody. (*C*) The hydroxyl group of the threonine at position 234 is essential for TRAF binding. The same amounts of GST protein and GST fusion proteins containing wild-type C17, T234A, T234S, and T234V were incubated with 293T cell extracts overexpressing Flu-tagged TRAF2 and TRAF3. The bound Flu-TRAF2 and Flu-TRAF3 were detected by Western blot analysis using anti-Flu antibody.

proteins T234S and T234V. Both TRAF2 and TRAF3 bound to the T234S mutant as strongly as wild-type C17, whereas the T234V mutation completely abolished the binding of both TRAF2 and TRAF3 (Fig. 2*C*). Serine is one methyl group shorter than threonine, whereas valine has a methyl group in the place of threonine's hydroxyl. Thus, our results demonstrate that the hydroxyl group of the threonine at position 234 of CD40 is essential for TRAF binding.

Binding of TRAF2 Correlates with CD40-Mediated NF-κB and SAPK Activation. CD40 has been reported to activate multiple signal transduction pathways, including ones involving NF-κB, SAPK, and STAT (10, 24, 25). The cytoplasmic tail of CD40 associates with multiple downstream signaling mediators such as TRAF2, TRAF3, and, possibly, Jak3. To determine the roles of TRAF2, TRAF3, and Jak3 in CD40-mediated NF-κB and SAPK activation, we created full-length human CD40 variants in the pBABEpuro retroviral vector to express the wild-type receptor; the Q232A mutant that specifically abolishes TRAF2 binding; the T234A mutant that abolishes TRAF2, TRAF3, and Jak3 binding (10); the V241A mutant that specifically abolishes TRAF3 association; and a deletion mutant lacking the cytoplasmic tail of the human CD40 receptor (ΔC). Also included was a P207A mutant that was reported to abolish the association of Jak3 with the CD40ct (10).

BI-141 murine T cell hybridoma cells were retrovirally infected with the human CD40 and its variants and then selected with puromycin. These cells were chosen as target cells because their low-basal NF-κB activity facilitates the detection of induction by gel-shift assay. The levels of expression of the exogenous receptors were found to be similar among the different cell lines as determined by FACScan analysis (data not shown).

To evaluate the specificity and contributions of TRAF2, TRAF3, and Jak3 in CD40-mediated signaling, the cell lines were stimulated with an anti-human CD40 mAb, G28.5, and activation of the SAPK and NF-κB signaling pathways were investigated. SAPK/JNK1 activity was assessed by an *in vitro* kinase assay that measures the kinase activity of immunoprecipitated JNK1 using GST-Jun as the substrate. Nuclear extracts were processed from the same samples to assess NF-κB activation by gel-shift analysis using an NF-κB-specific oligonucleotide probe. The induction of both the SAPK/JNK1

FIG. 3. CD40 mutants that retain TRAF2 binding are capable of signaling through the NF-κB and SAPK/JNK1 pathways upon activation. Twenty-five million BI141 cells were either untreated or treated with 1 μg/ml of the murine anti-human CD40 mAb, G28.5, for 30 min, at which point the samples were either processed for NF-κB-dependent gel-shift assays or for the SAPK-dependent phosphorylation of GST-Jun.

activity and NF-κB activity was evident in the wild-type, P207A, and V241A human CD40 variants, whereas no induction was apparent in the Q232A, T234A, and the cytoplasmic deletion mutants (Fig. 3). Similar assays also were conducted with the full-length human CD40 variants encoding the P230A, V231A, E233A, and L235A mutants. Like the Q232A mutant, which loses TRAF2 binding but retains TRAF3 binding, the mutants encoding for P230A, V231A, and E233A are unable to activate the SAPK and NF-κB pathways upon stimulation with G28.5, whereas the L235A mutant, which loses TRAF3 binding but retains TRAF2 binding, still can activate both signaling pathways (P.D. and G.C., unpublished results). Correlating binding specificity and activation, it appears that the roles of TRAF3 and Jak3 in SAPK and NF-κB signaling are minimal whereas the role of TRAF2 is critical.

Binding of TRAF2 Correlates with CD40-Induced ICAM-1 Up-Regulation. CD40 activation is known to result in the up-regulation of multiple cell-surface receptors such as CD23, ICAM-1, FAS, B7, and others (2). To determine the biological significance of the signaling mediated by TRAFs 2 and 3 and Jak3, we assayed for the ability of the human CD40 variants to up-regulate ICAM-1 expression in the murine B cell lymphoma cell line, WEHI 231. WEHI 231 cells were retrovirally infected with constructs expressing the human CD40 and its variants in the same manner as for the BI-141 cells and then selected with puromycin. FACS analysis of these infected WEHI 231 cell lines after overnight treatment with the anti-human CD40-specific G28.5 antibody showed up-regulation of ICAM-1 expression in the stable cell lines expressing the wild-type, P207A, and V241A human CD40 receptors (Fig. 4). However, cell lines expressing the Q232A, T234A, and cytoplasmic deletion mutants did not up-regulate ICAM-1 (Fig. 4). The levels of the anti-hCD40 antibody-induced ICAM-1 up-regulation in the WEHI 231 cells were similar to those induced by CD40L (data not shown), and these results were reproducible. Thus, CD40-mediated ICAM-1 up-regulation correlates with the binding of TRAF2, but not TRAF3 or Jak3, to the CD40ct.

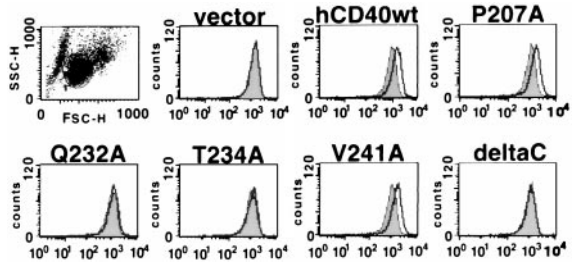

FIG. 4. CD40 mutants that retain TRAF2 binding are capable of up-regulating ICAM-1 upon stimulation. Murine Wehi 231 cells expressing wild-type and mutant variants of human CD40 were either untreated (shaded) or treated (dark outline) for 24 hr with 1 μg/ml of G28.5. The cells were stained with R-phycoerythrin-conjugated anti-murine ICAM-1 antibodies and analyzed by FACS.

CD40-Induced Up-Regulation of ICAM-1 Is NF-κB-Dependent. Our data suggest that TRAF2 signaling is crucial for NF-κB and SAPK activation through CD40 and that the signals generated by TRAF2 are crucial for at least one aspect of CD40 biology, namely, ICAM-1 induction. To further understand the role of NF-κB activation in CD40-mediated ICAM-1 up-regulation, we generated Daudi human B cell lymphoma lines that overexpressed a dominant-active inhibitor of NF-κBα (IκB-α), containing serine-to-alanine mutations at amino acid positions 32 and 36, that specifically prevents NF-κB activation (26). The cells were shown to express the mutant IκB at levels 2- to 3-fold higher than endogenous IκB-α and were unable to up-regulate NF-κB activity upon CD40 activation (H.H.L. and G.C., unpublished results). In examining the inducibility of ICAM-1 upon CD40 activation, it was found that CD40 ligation with G28.5 markedly up-regulated ICAM-1 expression on wild-type, parental Daudi cells and on control cells transfected with the empty expression vector (Fig. 5*A*). However, CD40-induced ICAM-1 up-regulation was abolished in the cells expressing the dominant-active IκB-α (Fig. 5*A*), supporting the importance of NF-κB signaling in CD40-mediated ICAM-1 up-regulation.

The transcriptional regulation of ICAM-1 by inflammatory cytokines has been studied extensively. Through mutational analysis of the ICAM-1 promoter, it was determined that the NF-κB-binding site present at −187 to −178 relative to the transcriptional start site is crucial for ICAM-1 up-regulation by TNF-α, lipopolysaccharide, phorbol 12-myristate 13-acetate, and interleukin 1 (21). To determine whether CD40-mediated up-regulation of ICAM-1 required similar cis elements, pGL2 luciferase reporters driven by either a 1.3-kb ICAM-1 5′ promoter fragment (extending from −1353 to −9 of the transcriptional start site, pGL 1.3) or a 1.3-kb promoter fragment with mutations in the NF-κB-binding site at −187 to −178 (from TGGAAATTCC to TctAgATTag, pGL 1.3κB−) were transiently transfected into A549 human lung carcinoma cells. The samples also were cotransfected with combinations of pBABEpuro CD40, pBABEpuro CD40L, pRCCMV IκB-dominant active construct, and empty vector. As a positive control, similar transfections also were carried out with the artificial NF-κB-dependent luciferase reporter construct, $(\kappa B)_3$-IFN-LUC (19). As expected, a strong induction was seen with the $(\kappa B)_3$-IFN-LUC reporter upon induction with CD40 and CD40L (Fig. 5*B*). A 3-fold luciferase induction was observed with the pGL 1.3 reporter (Fig. 5*B*); this induction was suppressed completely with cotransfection with the dominant-active IκB construct, consistent with the results in the Daudi cells (Fig. 5*A*). Induction of the luciferase activity, however, was absent in the pGL 1.3κB− samples (Fig. 5*B*), indicating that the NF-κB-binding site from −187 to −178 is also critical for CD40-mediated ICAM-1 induction.

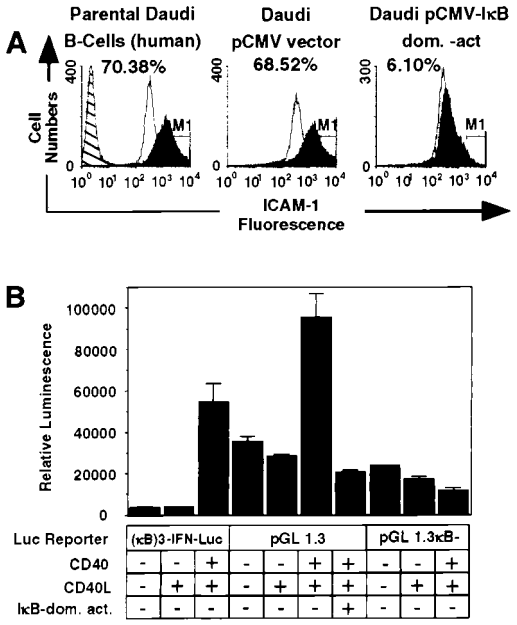

FIG. 5. CD40-induced expression of ICAM-1 is regulated through the NF-κB-dependent signal transduction pathway. (*A*) CD40-induced ICAM-1 up-regulation is inhibited by the overexpression of a dominant-active IκB mutant (pCMV IκB dom.-act.). FACS analysis of Daudi cells and Daudi transfectants (pCMV-IκB dom.-act. or pCMV vector) after 24 hr of culture with (shaded) or without (unshaded) CD40L stimulation. The hatched peak represents parent Daudi cells stained with a nonspecific control antibody. The *x* and *y* axes represent the relative intensity of ICAM-1 expression and relative cell numbers, respectively. The percentage of ICAM-1 induced cells is indicated in the upper portion of each histogram. (*B*) CD40-mediated up-regulation of a luciferase reporter driven by a 1.3-kb 5′ promoter region of ICAM-1 is dependent on NF-κB binding to a consensus site located at −187 to −178 relative to the transcriptional start site. A549 human lung carcinoma cells were transiently transfected with either the $(\kappa B)_3$-IFN-LUC reporter, the pGL2 1.3 luciferase reporter, or the pGL2 1.3κB- reporter, which has a mutated cis element between sites −187 and −178. Samples also were cotransfected with combinations of pBABEpuro hCD40WT, pBABEpuro mCD40L, pRCCMV IκB dominant-active construct, and empty vector. As a normalization factor, all samples were cotransfected with 1 μg of pCMVlacZ reporter, and the *y*-axis data points represent luciferase data after correcting for lacZ activity.

Our results thus provide a functional link from the CD40 receptor to ICAM-1 up-regulation in which oligomerization of the receptor activates TRAF2, which, in turn, activates NF-κB, a transcription factor that may directly be involved in the transcriptional activation of the ICAM-1 gene. The results, however, do not rule out the possibility that other components of TRAF2 signaling, such as SAPK activation, also are involved in ICAM-1 activation.

DISCUSSION

TRAF family proteins are major adapter molecules transducing signals from the TNF receptor family and other non-TNF receptors (11, 12, 15, 27). The cytoplasmic tail of CD40 has been reported to bind to at least four known TRAF proteins—TRAF2, TRAF3, TRAF5, and TRAF6—based on the yeast two-hybrid and *in vitro* GST pull-down assays (5–9). However, the relative affinities among these four TRAF proteins to the cytoplasmic tail of CD40 have not been determined. By constructing these TRAF cDNAs into the same epitope-tagged vector, we found with an *in vitro* binding assay that the interactions of TRAF5 and TRAF6 with the CD40ct are much weaker than those of TRAF2 and TRAF3, suggesting that TRAFs 2 and 3 are the main mediators of CD40 signaling.

Using alanine-scanning mutagenesis, we found that TRAF2 and TRAF3 binding to the CD40ct require different subsets of amino acid residues, even though both TRAFs bind strongly to a 17-aa domain within the CD40ct. Interestingly, both TRAF2 and TRAF3 require the threonine 234 residue of the CD40ct for their binding, and our results underscore the importance of the hydroxyl moiety at the 234 site. Phosphorylation at the same threonine 234 residue of CD40 has been reported (28), and its significance needs to be evaluated further.

Another level of signaling specificity arises from the different abilities of the TRAF proteins to activate downstream signal transduction pathways. Overexpression of TRAF2, TRAF5, and TRAF6 but not TRAF1, TRAF3, and TRAF4 in 293 cells activates both the NF-κB and SAPK pathways (7, 8, 11, 13, 16, 17). Embryonic fibroblast (EF) cells isolated from TRAF2 knockout mice failed to activate SAPK, but had only a delayed response in NF-κB activation after stimulation with TNF-α (29). The discrepancy between the results from transient transfection assays and those from knockout mouse

studies in TNF-α-induced activation of the NF-κB pathway could be due to either the existence of an alternative NF-κB activation pathway through the TNF receptors or redundancy of TRAF2 with other TRAF family members. Despite the fact that TRAF2 and TRAF3 have differing abilities to activate NF-κB and SAPK, interestingly, the phenotypes of TRAF2 knockout mice are similar to those of TRAF3 knockout mice, in that they all die about 2 weeks after birth and are depleted of both T and B lymphocytes (29, 30). Conversely, transgenic mice overexpressing a truncated form of TRAF2 have increased numbers of B cells, although cells isolated from these mice behave similarly to EF cells isolated from TRAF2 knockout mice in TNF-mediated NF-κB and SAPK activation (29, 31). Based on those previously published results, the roles of TRAF2 in NF-κB signaling and in B cell activation are still unclear.

Here, we took a different approach by using the results we obtained from alanine-scanning mutagenesis and making various stable cell lines expressing mutant CD40 that would abolish the binding of either TRAF2 or TRAF3. Although we cannot exclude the possible involvement of other TRAF proteins or even non-TRAF proteins in CD40-mediated NF-κB and SAPK activation, the results in both Figs. 1 and 3 suggest that TRAF2 may be involved in both the NF-κB and SAPK signal transduction pathways, at least in the case of CD40. The role of TRAF3 in CD40 signaling is not clear. It did not seem to be involved in CD40-mediated NF-κB and SAPK activation despite its high affinity for the cytoplasmic tail of CD40. However, studies using TRAF3 knockout mice indicated that TRAF3 is essential for T-dependent antigen responses, a function that is defective in CD40 knockout mice. Therefore, it will be interesting to find out whether TRAF3 or other TRAF family proteins can activate signal transduction pathways other than NF-κB and SAPK.

In the past several years, studies using various CD40-responsive cell lines have identified more than a dozen CD40-inducible genes, including those encoding for CD23, ICAM-1, B7.1, B7.2, Bcl-x, cdk4, cdk6, EGFR, A20, and major histocompatibility complex II (2, 32, 33). However, it was not clear which signal transduction pathways are responsible for particular downstream CD40-inducible genes. We took advantage of the human-specific CD40 mAb, G28.5, and determined the effects of various human CD40 mutants upon induction of CD40-mediated gene expression in murine B cells. Our results suggest that TRAF2 is not only important for CD40-mediated NF-κB and SAPK activation, but is also involved in CD40-mediated up-regulation of the ICAM-1 gene. To further determine whether the NF-κB or the SAPK pathways are required for ICAM-1 induction, we made stable B cell lines overexpressing a mutant IκB, which contains a serine-to-alanine mutation at the amino acid positions 32 and 36 of IκB. As a result, CD40 was unable to activate NF-κB and also was incapable of inducing ICAM-1 up-regulation, demonstrating that the NF-κB pathway is essential for CD40-mediated ICAM-1 induction. Our results emphasize the importance of the NF-κB pathway, but do not exclude the involvement of other signal transduction pathways such as SAPK or Jak/STAT in CD40-mediated ICAM-1 induction.

It has been reported that CD40 directly binds to Jak3 and mediates STAT3 activation (10). The same report also suggested that CD40-mediated ICAM-1 up-regulation is mediated through the Jak3/STAT3 pathway. However, we did not observe any differences in the abilities of ICAM-1 up-regulation between wild-type CD40 and the mutant CD40 carrying the P207A mutation, which was characterized previously as abolishing Jak3 association with the CD40ct. One possible explanation is that the mutations used previously, such as T234A, also abolish TRAF2 binding. However, further studies are required to confirm the role of the Jak3/STAT3 pathway in CD40-mediated ICAM-1 induction.

Upstream of the ICAM-1 promoter, there is an NF-κB-binding site that has been reported to mediate NF-κB-dependent ICAM-1 transcription (21). By using luciferase reporter genes driven by either the wild-type ICAM-1 promoter or the mutant ICAM-1 promoter carrying point mutations in the NF-κB-binding site, we found that the NF-κB-binding site in the upstream ICAM-1 promoter is critical for CD40-mediated up-regulation of the ICAM-1 gene, suggesting that the CD40-induced NF-κB activities may directly target the ICAM-1 promoter and be involved in transcriptional activation of the ICAM-1 gene. In combination with the existing data, we propose a CD40-mediated signal transduction pathway that cascades from CD40L to CD40 to TRAF2 to phosphorylation of IκB to activation of NF-κB and, finally, to ICAM-1.

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