Tumor Necrosis Factor Receptor-associated Factor 6 (TRAF6) Stimulates Extracellular Signal-regulated Kinase (ERK) Activity in CD40 Signaling Along a Ras-independent Pathway

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Summary

CD40 activates nuclear factor kappa B (NFκB) and the mitogen-activated protein kinase (MAPK) subfamily, including extracellular signal-regulated kinase (ERK). The CD40 cytoplasmic tail interacts with tumor necrosis factor receptor–associated factor (TRAF)2, TRAF3, TRAF5, and TRAF6. These TRAF proteins, with the exception of TRAF3, are required for NFκB activation. Here we report that transient expression of TRAF6 stimulated both ERK and NFκB activity in the 293 cell line. Coexpression of the dominant-negative H-Ras did not affect TRAF6-mediated ERK activity, suggesting that TRAF6 may activate ERK along a Ras-independent pathway. The deletion mutant of TRAF6 lacking the NH2-terminal domain acted as a dominant-negative mutant to suppress ERK activation by full-length CD40 and suppress prominently ERK activation by a deletion mutant of CD40 only containing the binding site for TRAF6 in the cytoplasmic tail (CD40Δ246). Transient expression of the dominant-negative H-Ras significantly suppressed ERK activation by full-length CD40, but marginally suppressed ERK activation by CD40Δ246, compatible with the possibility that TRAF6 is a major transducer of ERK activation by CD40Δ246, whose activity is mediated by a Ras-independent pathway. These results suggest that CD40 activates ERK by both a Ras-dependent pathway and a Ras-independent pathway in which TRAF6 could be involved.

CD40 is a cell-surface glycoprotein on B lymphocytes, dendritic cells, follicular dendritic cells, and thymic epithelial cells (1), and a member of the TNF-α receptor superfamily that includes 55- and 75-kD TNF receptors (TNFR1 and TNFR2, respectively), CD30 receptor, low-affinity nerve growth factor receptor, lymphotoxin β receptor (LTβR), and Fas antigen (1).

The cytoplasmic domain of the TNFR superfamily members lacks sequences indicative of catalytic activity, but is associated with a signal transducer, TNFR-α-associated factor (TRAF; reference 2). The cytoplasmic domain of human CD40 consists of 62 amino acids at positions 196–257 and is associated with TRAF2, TRAF3, TRAF5, and TRAF6 (3–9) and Janus kinase (Jak)3 (10); the membrane proximal region of the cytoplasmic tail of CD40 contains a proline-rich region at positions 202–209 that is crucial for Jak3 binding (10). TRAF6 binds to the NH2-terminal cytoplasmic tail of CD40 at positions 210–225, although the possibility can not be excluded that full association of TRAF6 with CD40 may also require the COOH-terminal part at positions 226–249 (9). TRAF2, TRAF3, and TRAF5 bind to the COOH-terminal cytoplasmic domain at positions 226–249 (9), containing a minimum element, designated TIMct, responsible for TRAF2 and TRAF3

*Abbreviations used in this paper: β-gal, β-galactosidase; aa, amino acids; DN, dominant negative; ERK, extracellular signal-regulated kinase; GST, glutathione-S-transferase; Jak, Janus kinase; JNK, c-jun NH2-terminal kinase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAPK/ERK-activating kinase; NFκB, nuclear factor kappa B; TRAF, TNFR-associated factor; TRAF-C, TRAF-COOH; TRAF-N, TRAF-NH2.
binding and signal transduction mediating nuclear factor kappa B (NFκB) activation (7).

Stimulation of CD40 results in activation of protein tyrosine kinases (PTKs), NFκB, the mitogen-activated protein kinase (MAPK), and Jak3/signal transducers and activators of transcription (STATs) (10–18), and it mediates critical biological effects in B cell growth, survival, and differentiation (19–27). It is known that TRAF2 and TRAF5 play a role in NFκB activation in signaling through CD40, as well as TNFR1, TNFR2, CD30, and lymphotixin β receptor (6–8, 28–32). TRAF6 participates in NFκB activation signaled by CD40 and IL-1 receptor (9, 33). The TRAF family is characterized by a homologous COOH-terminal TRAF-COOH (TRAF-C) domain, an α-helical TRAF-NH$_2$TRAF-N domain, and an NH$_2$-terminal RING finger with the exception of TRAF1 (2–6, 8, 9, 30, 33). The effector function of TRAF2 and TRAF5 toward NFκB activation is mediated by its NH$_2$-terminal RING finger domain (6, 8, 30), whereas that of TRAF6 is mediated by the RING finger and zinc fingers (9, 33).

It has been reported that TRAF2 stimulates c-Jun NH$_2$-terminal kinase (JNK) activity in TNFR1 signaling (34–36), leading to the idea that TRAF2 may also play a role in JNK activation by CD40 (15, 16). However, the signaling pathway coupling CD40 to extracellular signal-regulated kinase (ERK) activation has remained unknown. To investigate which TRAF proteins might participate in ERK activation, we have performed transient transfection experiments in the human embryonic kidney 293 cell line. In the present study, we demonstrate that TRAF6 plays a role as a signal transducer in ERK activation by CD40, probably along a Ras-independent pathway.  

### Results and Discussion

TRAF6 is a Signal Transducer of Both ERK and NFκB Activation. Overexpression of TRAF2, TRAF5, and TRAF6 results in its aggregation and causes activation of the NFκB pathway (37).

### Materials and Methods

**Cell Culture.** Human embryonic kidney cell line 293 was maintained in DME supplemented with 10% FCS, 200 mM l-glutamine, and penicillin/streptomycin.

**Plasmid Construction.** TRAF2, TRAF3, and human CD40 cDNA were obtained by the reverse transcription PCR (R-PCR) by use of messenger RNA purified from B cell lymphomas, WEHI231 and RAjI, with primers flanking the entire coding region, and then cloned into pMIKHygB, a gift from Dr. K. Maruyama (Tokyo Medical and Dental University, Tokyo, Japan). Deletion mutants of TRAF2 that lack a RING finger motif (amino acids [aa] 87–501) and the TRAF-C domain (aa 352–501) were constructed by PCR. Isolation of TRAF5 and TRAF6 cDNAs and construction of the expression vectors coding full-length TRAF5, full-length TRAF6, and the TRAF-C of TRAF6 were reported previously (9, 30). A deletion mutant of human CD40, designated CD40Δ226, that removes 32 aa at positions 226–257 from the cytoplasmic tail was previously described (9). Dominant-negative R-α1 (aa 1–258) was constructed by reverse transcription PCR, according to Schaap et al. (37). Dominant-negative N17Ras (as 38) and glutathione-S-transferase (GST) fusion ERK2 (pGSTM1) were provided by Dr. G.M. Cooper (Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA) and Dr. K. Takishima (National Defense Medical College, Saitama, Japan), respectively. Construction of a dominant-negative mutant of MAPK/ERK-activating kinase (MEK1), designated MEK1DN, and a constitutively activated mutant of MEK1, designated MEK1EE, was previously described (39). All cDNA fragments were subcloned into pMIKHygB.

ERK-K-dependent activator and reporter constructs were prepared according to Seth et al. (40, 41). In brief, the fusion of the GAL4 DNA-binding domain and the nuclear localization signal (aa 1–147) and NHR-terminal transcriptional activation region of c-Myc (aa 2–103) were constructed in vector pGBT9 (Clontech, Palo Alto, CA), and its HindIII-Sall fragment was digested and subcloned into pMIKHygB, designated pGAL4mycN. A firefly luciferase reporter containing two copies of the GAL4 binding site adjacent to a thymidine kinase promoter was constructed into pGL2 (Promega, Madison, WI), designated pGAL4tkLuc. A previous study by Seth et al. (41) showed that, in cotransfected cells with the GAL4–c-Myc fusion construct and luciferase reporter, the phosphorylation state of the NHR-terminal c-Myc transcription domain was increased in an ERK-dependent manner, resulting in an increase in the activity of the luciferase reporter gene (41). Therefore, this ERK-K-dependent activator and reporter system allows one to monitor ERK activity by measuring the luciferase reporter activity. NFκB-dependent luciferase constructs containing three copies of an NFκB-binding site from immunoglobulin κ-light chain enhancer and the thymidine kinase promoter was constructed into pGL2, pkBtkLuc. All cDNAs and constructs were confirmed by DNA sequencing.

In Vitro MAPK Assay. An in vitro kinase assay for endogenous ERK activity was performed as previously described (17). For estimating exogenous ERK2 kinase activity, 293 cells were cotransfected with 1 μg of GST–ERK2 and various amounts of plasmid constructs by the calcium phosphate coprecipitation method. The total amount of DNA was adjusted with empty vector at 10 μg. When 293 cells were cotransfected with 1 μg of CD40 cDNA, the cells were stimulated with anti-CD40 mAb (2 μg/ml) 24 h after transfection. In some experiments, cells were analyzed for the expression of CD40 on the surface by FACScan® (Becton Dickinson, Mountain View, CA) with FITC-conjugated anti–human CD40 mAb (5C3; Pharmingen, San Diego, CA). The cell lysates were incubated with glutathione-coupled Sepharose 4B (Pharmacia, Uppsala, Sweden) at 4°C for 1 h. The Sepharose beads were pelleted down by centrifugation and washed twice with lysis buffer and with Tris-buffered saline (10 mM Tris-HCl, pH 7.2, 100 mM NaCl) supplemented with 5 mM benzamidine and 1 mM NaVO$_4$. The GST–ERK2 immobilized on Sepharose was directly subjected to a kinase reaction as previously described (17).

Luciferase Assay. Cells were cotransfected with either full-length CD40 or CD40Δ226 and ERK-K-dependent activator and reporter plasmids consisted of 500 ng of pGAL4tkLuc and 100 ng of pGAL4mcyN, or NFκB-dependent plasmid of 20 ng of pkBtkLuc, 1 μg of β-actin β-galactosidase (β-gal), and various amounts of expression plasmids by the calcium phosphate coprecipitation method. Total amounts of DNA were adjusted with empty vector at 10 μg. The cells were stimulated with anti-CD40 mAb 24 h after transfection. The cells were lysed in PicoGene® Cell Culture Lysis Reagent LCP (Toyo Ink, Tokyo, Japan) 24 h after incubation, and luciferase activity was measured by use of a luciferase assay kit, PicoGene® (Toyo Ink), followed by normalization of transfection efficiency by β-gal activity.
signaling pathway, which could be similar to that induced by ligand-triggered receptor aggregation (6, 8, 9, 30, 33). Therefore, to investigate which TRAF proteins might participate in ERK activation, we cotransfected human embryonic kidney 293 cells with various TRAF expression vectors and an GST-ERK2 fusion construct. After transfection, the GST–ERK2 fusion protein was precipitated by use of glutathione Sepharose 4B from the cell lysate, and the ERK activity was analyzed by an in vitro kinase assay, with myelin basic protein (MBP) used as an exogenous substrate. As shown in Fig. 1A, transient expression of full-length TRAF6, but not TRAF2, TRAF3, or TRAF5, stimulated exogenous ERK activity approximately fivefold above the vector control, suggesting that transient expression of TRAF6 stimulates ERK activity in the 293 cell line.

ERK is known to phosphorylate target protein within the cytosol (42), whereas, under certain conditions, ERK translocates to the nucleus to phosphorylate the nuclear substrate (41, 43–45). To investigate the effect of various TRAF proteins on ERK activity in the nucleus, we used an ERK-dependent activator and reporter system, established by Davis and coworkers (40, 41, and see Materials and Methods). Human 293 cells were transiently transfected with various TRAF expression vectors, together with an ERK-dependent activator, a fusion construct of the GAL4 DNA-binding domain and the nuclear localization signal/NH2-terminal transactivation domain of c-Myc, and a luciferase reporter plasmid containing GAL4 binding sites adjacent to a thymidine

Figure 1. Transient expression of TRAF6-stimulated ERK activity in 293 cells. (A) Cells (10⁶) were transiently transfected with expression plasmid of TRAF proteins (5 μg each) together with GST-ERK2. GST-ERK2 fusion protein was precipitated by use of glutathione Sepharose 4B from cell lysates 48 h after transfection and subjected to in vitro kinase assay using MBP as a substrate. Arrow, phosphorylated MBP in the autoradiogram. (B) Cells (10⁶) were transiently transfected with 100 ng of ERK-dependent activator pGAL4MycN and 500 ng of the reporter pGAL4tkLuc (a) or 20 ng of the NFκB-dependent luciferase reporter pκBtkLuc (b), together with empty vector, full-length TRAF6 (3 or 5 μg), or the TRAF-C of TRAF6 (3 or 5 μg). Total amount of DNA was adjusted with empty vector at 10 μg. Luciferase activities were measured 48 h after transfection and normalized by β-gal expression. The extent of stimulation (relative activation) was calculated by comparison between luciferase activity given by the cells cotransfected with each TRAF expression plasmid and that of cells transfected with empty vector. (C) Cells (10⁶) were transiently cotransfected with either TRAF2, TRAF3, or TRAF3 (5 μg each) and ERK– (a) or NFκB–dependent luciferase reporter (b). Cell lysates were subjected to luciferase assay 48 h after transfection. The results shown in B and C were obtained from parallel experiments. For comparison, the extent of stimulation in ERK and NFκB by transient expression of TRAF6 (5 μg) in B is also shown in C. The mean value of two independent experiments (column) was calculated from a mean value of a single experiment in triplicate (closed circles). Arrowhead, phosphorylated MBP in the autoradiogram.

Figure 2. Inhibition of TRAF6-mediated ERK activity by dominant-negative mutant of Raf and MEK1, but not by Ras. Cells (10⁶) were cotransfected with ERK-dependent activator and reporter (A) or 500 ng of GST-ERK2 (B) and full-length TRAF6 (5 μg), together with 5 μg of N17Ras (DN Ras), DN-Raf, or MEK1DN (DN MEK). Cell lysates were prepared 48 h after transfection and subjected to luciferase assay (A) or in vitro kinase assay (B). The mean value of relative activity of ERK-dependent luciferase reporter (column) in four independent experiments was calculated from a mean value of a single experiment in triplicate (closed circles).
TRAF6 Stimulates ERK Activity in CD40 Signaling

TRAF6 is Involved in ERK Activation in CD40 Signaling. The truncated mutant of TRAF proteins that lack the RING finger or the RING finger and zinc fingers acts as a transdominant-negative mutant to suppress NFκB activation in signaling through TNFR1, TNFR2, IL-1R, CD30, and CD40 receptor (6, 8, 9, 28, 30, 31, 33), in which the dominant-negative mutant may act specifically by replacing the corresponding endogenous wild-type TRAF protein.

To investigate whether TRAF6 could be involved in ERK activation by CD40, we cotransfected 293 cells with the TRAF-C of TRAF6 and ERK-dependent activator and reporter, or GST-ERK, together with full-length CD40 cDNA. Because a previous report suggested that the NH2-terminal CD40 cytoplasmic tail at positions 210–225 could be essential for TRAF6 binding and could function for NFκB activation (9), the involvement of TRAF6 in ERK activation by CD40 was also investigated by transient transfection of the TRAF-C of TRAF6 and ERK-dependent activator and reporter, or GST-ERK, into 293 cells expressing a deletion mutant of CD40, CD40Δ246 (9), that removes the COOH-terminal cytoplasmic tail at positions 226–257. Previous results showed that TRAF6 interacts with the CD40Δ246 construct in vivo (9).

As shown in Fig. 3, A (a) and B, cross-linking of full-length CD40 and CD40Δ246 caused ERK activation approximately four- to sixfold above the level of unstimulated cells, comparable to the magnitude of the endogenous ERK activity that was transiently increased in 293 transfectants which constitutively expressed CD40 after stimulation with anti-CD40 mAb (data not shown). As shown in Fig. 3, A (a) and B both ERK-dependent reporter activity (Fig. 3 A) and the exogenous ERK kinase activity (Fig. 3 B) mediated by full-length CD40 were suppressed by coexpression of TRAF-C of TRAF6 in a dose-dependent manner to a maximum of 30–35% of inhibition, compatible with the notion that TRAF6-dependent and -independent pathways could be involved in ERK activation by CD40 (see below). Coexpression of the TRAF-C of TRAF6 caused prominent suppression of ERK activity mediated by cross-linking of CD40Δ246 in a dose-dependent manner, suggesting a major role of TRAF6 in ERK activation by CD40Δ246. It is not likely that the transdominant-negative mutant of TRAF6 nonspecifically diminished the activity of the ERK-dependent activator and reporter, because activation of the ERK-dependent reporter mediated by coexpression of catalytically active MEK1 (39) in the 293 cell line was not prevented by coexpression of TRAF-C of TRAF6 (data not shown).

In control experiments, transient expression of the TRAF-C of TRAF6 caused prominent suppression of the activity of cotransfected NFκB-dependent reporter mediated by CD40Δ246 in the 293 cell line (Fig. 3 C b), consistent with previous reports (9). In contrast to ERK activation, the activation of NFκB activity by full-length CD40 was greater than that by CD40Δ246, and ~50% of the activity was suppressed by coexpression of the TRAF-C of TRAF6 (Fig. 3 C a). Because of a similar frequency and the level of expression of full-length CD40 and CD40Δ246 in the 293 cell line in transient transfection assays (data not shown), the results seem compatible with the notion that distinct regions of the CD40 cytoplasmic domain could be required for full activation of NFκB in the 293 cell line.
Figure 3. Transient expression of TRAF-C of TRAF6 caused inhibition of ERK and NFκB activity mediated by cross-linking of CD40. Cells (10⁶) were transiently cotransfected with ERK-dependent activator and reporter (A), GST-ERK2 fusion construct (B), or NFκB-dependent reporter (C) and either 1 μg of full-length CD40 (a) or CD40Δ246 (b), together with 1 μg of TRAF-C domain of TRAF6. Total amount of DNA was adjusted with empty vector at 10 μg. Cells were stimulated with anti-CD40 mAb 24 h after transfection. After 24 h incubation, ERK activity was measured by luciferase assay (A) or in vitro kinase assay (B). Arrowhead, phosphorylated MBP in the autoradiogram is indicated by an arrowhead. NFκB activity was measured by luciferase assay (C). Relative activation of ERK and NFκB in each transfection was calculated as described above. The mean value of two to three independent experiments (column) was calculated from a mean value of a single experiment in triplicate (closed circle).

Figure 4. ERK activation by cross-linking of full-length, but not that by CD40Δ246, was prominently suppressed by dominant negative mutant of Ras. Cells (10⁶) were cotransfected with ERK- (A) or NFκB-dependent reporter (B) and either 1 μg of full-length CD40 (a) or CD40Δ246 (b), together with 5 μg of N17Ras, DNRaf, and MEKDN. Total amount of DNA was adjusted with empty vector at 10 μg. Cells were stimulated with anti-CD40 mAb 24 h after transfection. After 24 h incubation, cell lysates were subjected to luciferase assay. Relative activation of ERK and NFκB in each transfection was calculated as described above. The mean value of two to three independent experiments (column) was calculated from a mean value of a single experiment in triplicate (closed circle).
CD40 activates ERK by the Ras-dependent Pathway and Ras-independent Pathway in which TRAF6 Could Be Involved. To investigate the role of Ras, Raf-1, and MEK in ERK activation by CD40, we cotransfected 293 cells with the ERK-dependent activator and reporter, or NFkappaB-dependent reporter as a control, and with N17Ras, DN Raf, or MEK1DN, together with either full-length CD40 or CD40Δ246. The experiments were performed in parallel with some of the experiments depicted in Fig. 2 A. Co-transfection of N17Ras as, DN Raf, and MEK1DN caused ~70% inhibition of ERK activity mediated by cross-linking of full-length CD40 (Fig. 4 A). In contrast, coexpression of N17Ras as and MEK1DN did not affect NFkappaB activation by both full-length CD40 and CD40Δ246, whereas DN Raf marginally suppressed NFkappaB activation (Fig. 4 B). In conjunction with the previous observation indicating activation of the Ras-mediated pathway by CD40 stimulation in B cells (52), the results support the possibility that the Ras-Raf-MEK-mediated pathway could be involved in ERK activation, but not NFkappaB, by cross-linking of CD40. Transient expression of N17Ras as resulted in ~30% inhibition of ERK activity mediated by CD40Δ246, compatible with the notion that TRAF6 is a major transducer of ERK activation by CD40Δ246, whose activity is mediated by a Ras-independent pathway.

Taken together, these results support the possibility that CD40 may activate ERK via Ras-dependent and -independent pathways in which TRAF6 could be involved. The inhibition by a dominant-negative mutant of MEK1 in ERK activity by full-length CD40 was more prominent than in ERK activity mediated by TRAF6 and by CD40Δ246, in which TRAF6 may play a major role. Because the MEK isoforms MEK1 and MEK2 are an ERK-specific kinase (53), these results might imply that preferential usage of MEK isoforms would differ in TRAF6-dependent and -independent pathways in CD40 signaling. In this context, it has been suggested that the signaling pathways leading to activation of MEK isoforms appear to differ, and that each MEK isoform could be preferentially used in a particular external stimulus in a certain type of cell (54–56). Further study is needed to clarify the signaling pathway coupling TRAF6 to ERK activation.

Given that full ERK activity is stimulated through TRAF6-dependent and -independent pathways in CD40 signaling, a similar extent of ERK activation by full-length CD40 and CD40Δ246 in the 293 cell line led to the idea that ERK activity could be regulated in full-length CD40 signaling in 293 cells, probably via activation of MAP kinase phosphatase or downregulation of MEK activity (57–60). In contrast to ERK activation in murine splenic resting B cells (17, 18), it was reported that CD40 stimulation did not cause ERK activation in human tonsil B cells (15, 16). Whether ERK activation is regulated in CD40 signaling, depending on the maturation stage of B cells, would be worth analyzing.

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