THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 21, Issue of May 22, pp. 13353–13358, 1998

Printed in U.S.A.

CD27, a Member of the Tumor Necrosis Factor Receptor Superfamily, Activates NF-κB and Stress-activated Protein Kinase/c-Jun N-terminal Kinase via TRAF2, TRAF5, and NF-κB-inducing Kinase*

(Received for publication, September 11, 1997, and in revised form, March 12, 1998)

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CD27 is a member of the tumor necrosis factor (TNF) receptor superfamily and is expressed on T, B, and NK cells. The signal via CD27 plays pivotal roles in T-T and T-B cell interactions. Here we demonstrate that overexpression of CD27 activates NF-κB and stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK). Deletion analysis of the cytoplasmic domain of CD27 revealed that the C-terminal PIQEDYR motif was indispensable for both NF-κB and SAPK/JNK activation and was also required for the interaction with TNF receptor-associated factor (TRAF) 2 and TRAF5, both of which have been implicated in NF-κB activation by members of the TNF-R superfamily. Co-transfection of a dominant negative TRAF2 or TRAF5 blocked NF-κB and SAPK/JNK activation induced by CD27. Recently, a TRAF2-interacting kinase has been identified, termed NF-κB-inducing kinase (NIK). A kinase-inactive mutant NIK blocked CD27-, TRAF2-, and TRAF5-mediated NF-κB and SAPK/JNK activation. These results indicate that TRAF2 and TRAF5 are involved in NF-κB and SAPK/JNK activation by CD27, and NIK is a common downstream kinase of TRAF2 and TRAF5 for NF-κB and SAPK/JNK activation.

CD27 is a member of the tumor necrosis factor receptor (TNF-R)[1] superfamily and is expressed on T, B, and NK cells as a disulfide-linked homodimer (1). CD27 ligand (CD70) belongs to the TNF superfamily and is expressed on the surface of activated T and B cells. Cross-linking of CD27 along with a suboptimal dose of phytohemagglutinin, phorbol 12-myristate 13-acetate, anti-CD2, or anti-CD3 antibodies resulted in vigorous proliferation of T cells, indicating that CD27 transmits a co-stimulatory signal in T cells (2). On the other hand, ligation of CD27 on B cells enhanced IgG production (3). These studies implicated the important roles of CD27/CD70 interaction in immunoregulation through T-T and T-B cell interactions. A recent report also demonstrated a critical role of CD27/CD70 interaction in T cell development (4). Although biological function of CD27/CD70 interaction has been extensively investigated, the mechanism by which CD27 transmits the signal has been largely unknown, except for a previous study, which demonstrated involvement of the protein tyrosine kinase cascade (2).

TNF receptor-associated factors (TRAFs) have emerged as signal transducers for some members of the TNF-R superfamily (5–10). All TRAFs, except for TRAF1, are composed of N-terminal zinc RING finger, multiple zinc fingers, coiled-coil, and C-terminal receptor binding (TRAF) domains (5, 6, 9–11). Whereas an N-terminal RING finger domain of TRAF2, TRAF5, and TRAF6 is responsible for NF-κB activation, the TRAF and coiled-coil domains are required for homo- and heterodimerization and receptor association (5, 6, 9, 10, 12). With the exception of TRAF4, all other TRAFs have been shown to interact directly with the non-death domain receptors, CD30, CD40, TNFR80, lymphotoxin-βR (LT-βR), and interleukin-1R (9, 10, 12–16). TRAF2 has been shown to interact indirectly with death domain receptors, TNFR60 and death receptor 3, via the adapter molecule TRADD (17–19). TRAF2, TRAF3, and TRAF5 also interact with latent infection membrane protein 1, the transforming protein of Epstein-Barr virus (11). TRAF2 also participates in the activation of stress-activated kinase (SAPK)/c-Jun N-terminal kinase (JNK) induced by TNF (20–22). TRAF3 and TRAF5 are involved in CD23 up-regulation by CD40 (6, 16). TRAF3 has been also shown to be involved in apoptosis by LT-βR (23). The mechanism by which TRAFs activate NF-κB remains to be solved. Recently, a serine/threonine kinase was identified that interacts with TRAF2 and activates NF-κB, named NF-κB-inducing kinase (NIK) (24). However, the functional role of NIK in TRAF5- or TRAF6-mediated NF-κB activation is uncertain.

CD27 is functionally similar to other co-stimulatory recep-

* This work was supported by grants from the Ministry of Education, Science, and Culture and the Ministry of Health, Japan. and the Uehara Memorial Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: TNF, tumor necrosis factor; TNF-R, TNF receptor; TRAF, TNF receptor-associated factor; LT-βR, lymphotoxin-β receptor; HEK, human embryonic kidney; HVEM, herpesvirus entry mediator; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase; NIK, NF-κB-inducing kinase; EMSA, electrophoretic mobility shift assay; mAb, monoclonal antibody; HA, hemagglutinin; PCR, polymerase chain reaction.

tors OX40, 4-1BB, and the herpesvirus entry mediator (HVEM) (25). HVEM was recently shown to interact with TRAF2 and TRAF5 (26, 27). Here we demonstrate that CD27 activates both NF-κB and SAPK/JNK, which are mediated by TRAF2 and TRAF5. We also demonstrate that a kinase-inactive mutant of NIK inhibited TRAF2-, TRAF5-, and CD27-mediated NF-κB and SAPK/JNK activation. These results indicated a crucial role of TRAF2 and TRAF5 in CD27 signaling and that NIK is a common downstream kinase of TRAF2 and TRAF5 for NF-κB and SAPK/JNK activation.

MATERIALS AND METHODS

Reagents and Cell Line—Biotin-conjugated monoclonal antibody (mAb) to Flag (M2) (Kodak), anti-hemagglutinin A (HA) (12CA5) (Boehringer Mannheim), horseradish peroxidase-conjugated rabbit anti-mouse IgG (Zymed), rabbit antibodies to NF-κB subunits, p50 and p65 (Santa Cruz Biotechnology) were obtained from the indicated commercial sources. The anti-human CD27 mAb (1A4) has been described previously (28). The human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Expression Vectors—Flag-tagged expression vectors for TRAF2 (Dr. T. Watanabe, Institute of Medical Science, University of Tokyo), TRAF3 (Dr. G. Mosialos, Harvard Medical School), TRAF5, and TRAF6 were constructed by inserting each cDNA into pCR-Flag vector, which has two copies of Flag tag sequence downstream of cytomegalovirus promoter in pCR-3 (Invitrogen). Deletion mutants of ΔTRAF2-(272–501) and ΔTRAF5-(233–558) were amplified by polymerase chain reaction (PCR) using primers corresponding to the numbered amino acids. The PCR products were ligated directly into pCR-Flag vector. The C-terminal deletion mutants of human CD27 (Δ5, Δ8, Δ10, and Δ16) were made by PCR, and the appropriate PCR fragments were inserted into pCR-3 vector. pCR-CD30 (15) (Dr. T. Watanabe, Institute of Medical Science, University of Tokyo), pCDM8-CD40 (29) (Dr. H. Kikutani, Research Institute for Microbial Disease, University of Osaka), pcDNA-LT-βR (9), pcDNA-NIK, pCR-Flag-NIK, pcDNA-NIK-KM(KK429–430AA), and pCR-Flag-NIK-KM(KK429–430AA) (24), and pSRα-HA-SAPK (30) (Dr. E. Nishida, Kyoto University) were described previously. Recombinant GST-c-Jun-(1–79) (Dr. E. Nishida, Kyoto University) was expressed and purified as described previously (31).

Electrophoretic Mobility Shift Assay (EMSA)—HEK293 cells (4×10^6) were plated in 100-mm dishes. The following day the cells were transfected with 5 μg of pCR-CD27 or an empty vector using LipofectAMINE reagent (Life Technologies, Inc.) according to a manufacturer's instruction. After 36 h, nuclear extracts were prepared from transfected cells, and EMSA was performed as described previously (9).

NF-κB-dependent Reporter Assays—HEK293 cells (1×10^6) were plated in 35-mm dishes. The following day the cells were transfected using LipofectAMINE. Transfections included 50 ng of β-actin-β-galactosidase (Dr. K. Yokota, NIH, Japan), β-actin promoter-driven β-galactosidase expression plasmid to normalize for transfection efficiency, together with 100 ng of reporter plasmid and various amounts of each expression vector. Total DNA (1 μg) was kept constant by supplementation with pCR-3. A reporter plasmid, 3x-κB-tk-luc, has three repeats of the NF-κB site upstream of a minimal thymidine kinase promoter and a luciferase reporter gene in pGL-2 vector (Promega) (32) (Dr. M. Kashiwada, NIH, Japan). After 24 h, the cells were harvested in phosphate-buffered saline and lysed in luciferase lysis buffer, LC-β (Piccagene). The lysates were assayed for luciferase and β-galactosidase activities using a luminometer (Berthold).

Co-immunoprecipitation and Western Blotting—HEK293 cells (1.5×10^6) were plated in 60-mm dishes and transfected with various expression vectors using LipofectAMINE. After 24–36 h, the cells were washed in ice-cold phosphate-buffered saline and lysed for 30 min on ice in 1 ml of a lysis buffer containing 1% Nonidet P-40, 50 mM HEPES (pH 7.3), 250 mM NaCl, 2 mM EDTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride. Cellular debris was removed by centrifugation, and the supernatant was precleared with protein A-conjugated beads (Bio-Rad) for 1 to 2 h. Cleared lysates were incubated with anti-CD27 mAb (1A4) for 1 h at 4 °C. After the addition of protein A beads, the lysates were rotated at 4 °C for 1 h. The beads were washed three times with the lysis buffer, and bound proteins were eluted with 1% SDS sample buffer, subjected to 10% SDS-polyacrylamide gel electrophoresis, and then blotted onto polyvinylidene difluoride membrane (Millipore). Expression of transfected constructs was verified by immunoblotting of aliquots of cell lysates. Flag-tagged TRAFs were detected using biotin-conjugated anti-Flag mAb followed by incubation with avidin-biotin complex (Vectastain) and enhanced chemiluminescence (ECL) Western Blotting Detection System (Amersham Pharmacia Biotech) according to the instructions of the manufacturer. Expression of full-length and deletion mutants of CD27 was detected using anti-CD27 mAb followed by horseradish peroxidase-conjugated rabbit anti-mouse Ig and ECL.

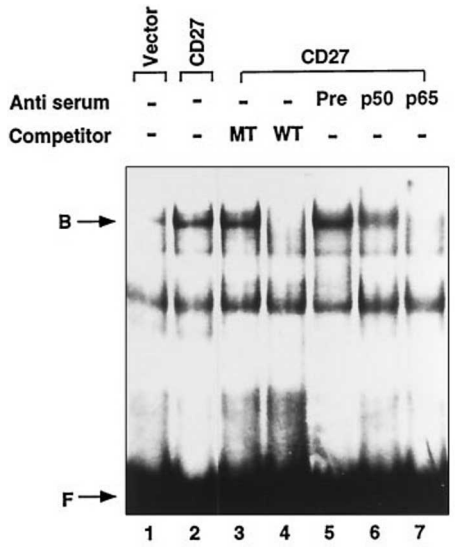

FIG. 1. **NF-κB activation induced by CD27.** HEK293 cells (4×10^6) were transfected with 5 μg of pCR control (*lane 1*) or full-length CD27 (*lane 2*). Nuclear extracts were prepared 24 h after transfection, and 5 μg of the nuclear extracts were incubated with a radiolabeled double-stranded κB oligonucleotide. Unlabeled competitor oligonucleotide containing either κB oligonucleotide (*WT*) (*lane 4*) or random oligonucleotide (*MT*) (*lane 3*) was added at a 50-fold molar excess. For supershift assays, reaction mixtures were incubated with 1 μl of either preimmune serum (*lane 5*), anti-p50 (*lane 6*), or anti-p65 (*lane 7*) serum. *B* and *F* indicate the position of the bound and free fraction, respectively.

SAPK/JNK Assay—Twenty-four hours after the transfection with the indicated expression vectors together with pSRα-HA-SAPK, HEK293 cells were lysed in 1 ml of a lysis buffer containing 1% Triton X-100, 20 mM HEPES (pH 7.3), 150 mM NaCl, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 0.1 mM sodium orthovanadate. Lysates were then clarified by centrifugation at 15,000 rpm for 5 min. The supernatants were immunoprecipitated with anti-HA mAb (12CA5) for 1 h at 4 °C. After the addition of protein A-conjugated beads, the lysates were incubated for an additional 1 h. The immunoprecipitates were washed three times with the lysis buffer and twice with a kinase buffer containing 20 mM HEPES (pH 7.3), 20 mM $MgCl_2$, 20 mM $MnCl_2$, 1 mM EDTA, 1 mM NaF, 0.1 mM sodium orthovanadate, 1 mM dithiothreitol and subjected to *in vitro* kinase assay. The immunoprecipitates were incubated with 1 μg of GST-c-Jun-(1–79) and [γ-^{32}P]ATP (10 μCi) in the kinase buffer for 20 min at 30 °C. The reaction was stopped by the addition of Laemmli's sample buffer. Phosphorylated proteins were subjected to 12% SDS-polyacrylamide gel electrophoresis, visualized by autoradiography, and quantified by using an image analyzer (Fujix, BAS2000). In all cases, expression of the transfected proteins was verified by immunoblotting of aliquots of the cell lysates.

RESULTS

Overexpression of CD27 Activates NF-κB—Transient transfection of HEK293 cells with full-length CD27 activated a significant amount of NF-κB as measured by DNA binding activity (Fig. 1). Supershift assay with antibodies against each component of the NF-κB complex demonstrated that this complex was mainly composed of p50/p65 subunits.

To delineate the functional domain of CD27 for NF-κB activation, we constructed a series of C-terminal deletion mutants (Fig. 2*A*) and performed a reporter gene assay after transfection into HEK293 cells. As expected from the result of EMSA, full-length human CD27 increased NF-κB-dependent lucifer-

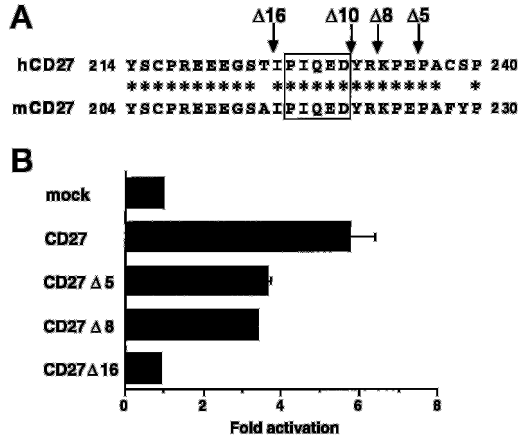

FIG. 2. **Delineation of the region required for NF-κB activation in the cytoplasmic domain of CD27.** *A,* computer alignment of the C-terminal region of murine and human CD27. The alignment was performed with GCG software. Conserved residues are indicated with *asterisks*. The putative core sequence of TRAF binding is *boxed*. The C-terminal ends of CD27 mutants (Δ5, Δ8, Δ10, and Δ16) are indicated by the *arrows*. *B,* PIQEDYR motif is required for NF-κB activation. HEK293 cells were transiently co-transfected with 3x-κB-tk-luc reporter gene plasmid with 1 μg of the indicated expression vectors for full-length or deletion mutants of CD27. After 24 h the cells were collected, and luciferase activity was determined for each sample, and the values were normalized to the expression of β-galactosidase. The level of induction in luciferase activity is indicated as compared with cells transfected with the control vector. The data represent one of three independent experiments with similar results. Values are shown as the mean ± S.D. of triplicate samples.

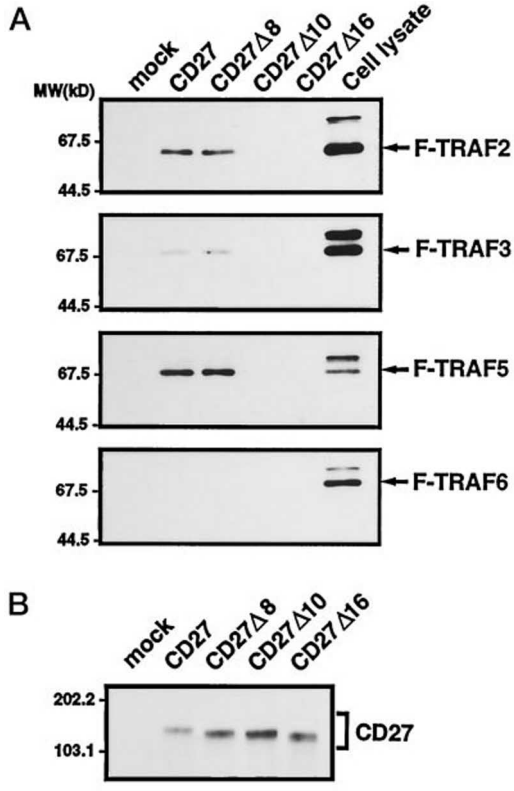

FIG. 3. **Binding of CD27 to TRAF2, TRAF3, or TRAF5.** A, HEK293 cells were co-transfected with full-length or deletion mutants of CD27 and Flag-TRAF2, Flag-TRAF3, Flag-TRAF5, or Flag-TRAF6. After 24 h, cell lysates were prepared and immunoprecipitated with anti-CD27 mAb. Co-immunoprecipitated TRAFs were detected by immunoblotting with anti-Flag mAb. Expression of Flag-TRAFs (*A*) and deletion mutants of CD27 (*B*) was verified by immunoblotting of the total lysates. The positions of molecular mass standards are indicated at the *left*.

ase expression approximately 6–7-fold over the level seen in vector-transfected cells (Fig. 2*B*), whereas C-terminal deletion mutants CD27Δ5 and CD27Δ8 exhibited only slight decrease in activation of NF-κB compared with the full-length CD27. By contrast, CD27Δ16 failed to activate NF-κB (Fig. 2*B*), suggesting that amino acids 225–232 are required for NF-κB activation. This region contains the sequence PIQED that is similar to the TRAF binding sequence, with a consensus sequence P*X*Q*X*(T or S) found in several other receptors, including CD30, CD40, or latent infection membrane protein 1 (13, 15, 33). To determine if this region of CD27 was sufficient for supporting TRAF binding, HEK293 cells were transiently co-transfected with Flag-TRAFs and full-length or mutant CD27 cDNAs. Flag-TRAF2 and Flag-TRAF5 were efficiently co-precipitated with CD27 and CD27Δ8 but not with CD27Δ10 or CD27Δ16 (Fig. 3*A*), consistent with their ability to activate NF-κB. That CD27Δ10 failed to interact with TRAFs indicated that PIQED sequence in CD27 is not sufficient to support the interaction in a cellular context. Flag-TRAF3 only weakly interacted with CD27 and Flag-TRAF6 did not bind at all. Immunoblot analysis of the total cell lysates demonstrated that the expression level of each TRAF (Fig. 3*A*) and CD27 mutant (Fig. 3*B*) was equivalent. These results indicated that CD27 interacts with TRAF2 and TRAF5, and the PIQEDYR motif in the cytoplasmic region is responsible for TRAF binding and NF-κB activation.

A Dominant Negative TRAF2 or TRAF5 Block NF-κB Activation Induced by CD27—To determine whether TRAF2 and TRAF5 are involved in NF-κB activation by CD27, we tested whether N-terminal truncated mutants of TRAF2 and TRAF5 would act as dominant negative mutants based on previous results that showed that the zinc binding domain of TRAF2 and TRAF5 is required for NF-κB activation (9, 12). Co-transfection of CD27 and truncated TRAF2(ΔTRAF2:272–499) or TRAF5(ΔTRAF5:233–558) blocked NF-κB activation as revealed by the reporter assay (Fig. 4*A*), suggesting that these two TRAFs are required for NF-κB activation by CD27.

Involvement of NIK in TRAF5- and CD27-mediated NF-κB Activation—NIK has been identified as a TRAF2-interacting kinase (24) and, thus, a likely candidate for mediating NF-κB activation by CD27. Co-transfection of a kinase-inactive mutant NIK (NIK-KM) with TRAF2, TRAF5, or CD27 blocked NF-κB activation (Fig. 4*B*). In addition, we also observed a dominant negative effect of NIK-KM on TRAF6-, CD30-, CD40-, and LT-βR-mediated NF-κB activation (Fig. 4*B*). These results indicated that NIK is a common downstream kinase of TRAF2, TRAF5, and TRAF6 for NF-κB activation in HEK293 cells.

CD27 Activates SAPK/JNK Pathway via TRAF2 and TRAF5—It has been shown that ligation of TNFR60 activated SAPK/JNK pathway, and this activation is dependent on TRAF2 (20–22). Together the data suggests that TRAF2 interacts with CD27 and thus CD27 may also activate SAPK/JNK pathway. To test this, HEK293 cells were co-transfected with HA-tagged SAPK and the C-terminal deletion mutants of CD27. The extent of SAPK/JNK activity was determined by immunoprecipitation of SAPK/JNK followed by *in vitro* kinase assay using GST-c-Jun-(1–79) as a substrate. Full-length CD27, CD27Δ5, and CD27Δ8, but not CD27Δ16, induced a 2.3- to 5.4-fold increase in SAPK/JNK activity (Fig. 5*A*). Overexpression of TRAF2 or TRAF5, but not TRAF3, also increased SAPK/JNK activity (Fig. 5*B*). These results indicated that CD27 activates SAPK/JNK pathway, and this activation is probably mediated by TRAF2 and TRAF5. This hypothesis was confirmed by the demonstration that N-terminal deletion mutants of TRAF2 or TRAF5 partially blocked SAPK/JNK activa-

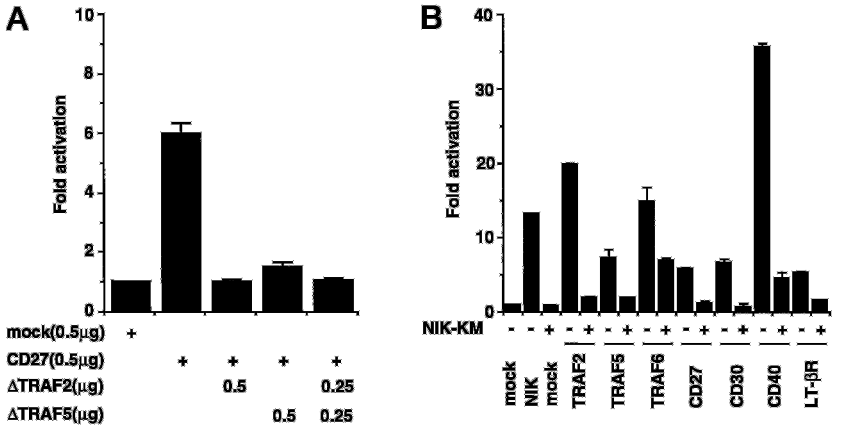

FIG. 4. **Involvement of TRAFs and NIK in CD27-mediated NF-κB activation.** *A*, N-terminal deletion mutants of TRAF2 or TRAF5 blocked NF-κB activation by CD27. 293 cells were co-transfected with 100 ng of 3x-κB-tk-luc reporter gene plasmid, 50 ng of β-actin-β-galactosidase, and the indicated amounts of expression plasmids. *B*, NIK-KM blocks NF-κB activation by TRAF2, TRAF5, TRAF6, CD27, CD30, CD40, and LT-βR. HEK293 cells were co-transfected with 100 ng of 3x-κB-tk-luc reporter gene plasmid, 50 ng of β-actin-β-galactosidase, and 0.5 μg of indicated expression vectors. Data are shown as the mean ± S.D. of triplicate samples and represent one of three independent experiments with similar results.

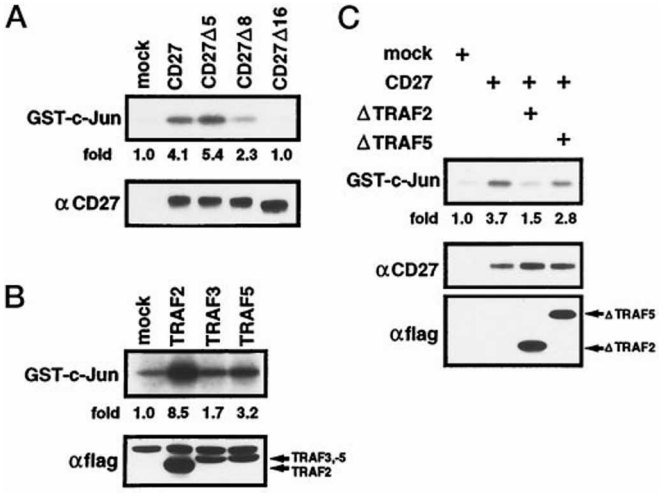

FIG. 5. **CD27 activates SAPK/JNK via TRAF2 and TRAF5 SAPK/JNK activation by CD27 (*A*), TRAF2 and TRAF5 (*B*).** HEK293 cells were co-transfected with 0.5 μg of HA-SAPK and 1.5 μg of CD27, deletion mutants of CD27, Flag-TRAF2, Flag-TRAF3, or Flag-TRAF5. HA-SAPK was immunoprecipitated, and kinase activity was assayed as described under "Materials and Methods." Phosphorylation of GST-c-Jun-(1–79) was quantified by an image analyzer, and the fold increase in the kinase activity is indicated below the autoradiograms. The expression levels of CD27 and its deletion mutants, Flag-TRAF2, Flag-TRAF3, and Flag-TRAF5 were monitored by immunoblotting with anti-CD27 mAb (*A*) or anti-Flag mAb (*B*). The positions of Flag-TRAFs are indicated at the *right*. *C*, inhibition of CD27-mediated SAPK/JNK activation by N-terminal deletion mutants of TRAF2 (ΔTRAF2) or TRAF5 (ΔTRAF5). HEK293 cells were co-transfected with 0.5 μg HA-SAPK and 1 μg of CD27 along with or without 0.5 μg of Flag-ΔTRAF2 or Flag-ΔTRAF5. HA-SAPK was immunoprecipitated, and *in vitro* phosphorylation of GST-c-Jun-(1–79) was performed as in *A* and *B*. The expression levels of CD27, Flag-ΔTRAF2, and Flag-ΔTRAF5 were determined by immunoblotting with anti-CD27 or anti-Flag mAb. The positions of Flag-ΔTRAF2 and Flag-ΔTRAF5 are indicated at the *right*.

tion by CD27 (Fig. 5*C*), indicating that TRAF2 and TRAF5 are signaling molecules for SAPK/JNK activation as well as for NF-κB activation by CD27.

NIK Activates SAPK/JNK and Is Involved in CD27-, TRAF2-, and TRAF5-mediated SAPK/JNK Activation—Considering that NIK is structurally related to mitogen-activated protein kinase kinase kinase, we tested whether NIK activates SAPK/JNK. Overexpression of NIK, but not a kinase-inactive mutant NIK (NIK-KM), substantially increased SAPK/JNK activity to 6.9-fold compared with the vector-transfected cells (Fig. 6*A*). We next examined the effect of NIK-KM on SAPK/JNK activation by CD27, TRAF2, and TRAF5. As shown in Fig. 6, *B* and *C*, co-transfection of NIK-KM partially inhibited CD27-, TRAF2-, and TRAF5-mediated SAPK/JNK activation, suggesting that NIK is a common downstream kinase for SAPK/JNK activation as well as for NF-κB activation, induced by these molecules.

DISCUSSION

Accumulating data have indicated important roles of TRAFs in signaling through certain members of the TNF-R superfamily. Here, we demonstrated that CD27 associates with TRAF2 and TRAF5, and these TRAFs are implicated in NF-κB and SAPK/JNK activation by CD27. Our results suggest that other members of the TNF-R superfamily, such as 4-1BB and OX40, may also interact with TRAFs and activate NF-κB and SAPK/JNK pathways. We also demonstrated that a kinase-inactive mutant NIK blocked TRAF5- and TRAF6-mediated NF-κB activation, indicating that NIK is a common downstream kinase of TRAF2, TRAF5, and TRAF6 for NF-κB activation. Previous studies (20, 34) implied that mitogen-activated protein kinase/ERK kinase kinase-1 may be responsible for TNF- and TRAF-mediated SAPK/JNK activation. Here we demonstrated that NIK also activates SAPK/JNK pathway and a kinase-inactive mutant NIK partially inhibited SAPK/JNK activation by TRAF2, TRAF5, and CD27. The dominant negative effect of NIK-KM on SAPK/JNK activation by CD27 and TRAFs appeared to be weaker than that on NF-κB activation. These results suggest that other mitogen-activated protein kinase kinase kinases, such as mitogen-activated protein kinase/ERK kinase kinase-1, could compensate the dominant negative effect of NIK-KM on SAPK/JNK activation. The mechanism by which NIK activates SAPK/JNK pathway is currently under investigation. A previous study showed that CD27 cross-linking induced tyrosine phosphorylation of several signal transducing molecules including ZAP70, and the treatment with a Src family kinase inhibitor, herbimycin, completely blocked co-stimulatory signal through CD27 (2). Collectively, these data suggest that CD27 activates two distinct signaling pathways, one is protein tyrosine kinase-dependent and another is TRAF-dependent pathway, the latter of which activates NF-κB and SAPK/JNK.

To date, it has been shown that CD27, CD30, CD40, LT-βR, and HVEM interact with both TRAF2 and TRAF5 (9, 15, 16, 26, 27). A common consequence of signaling through these receptors is the activation of NF-κB that can now be explained by

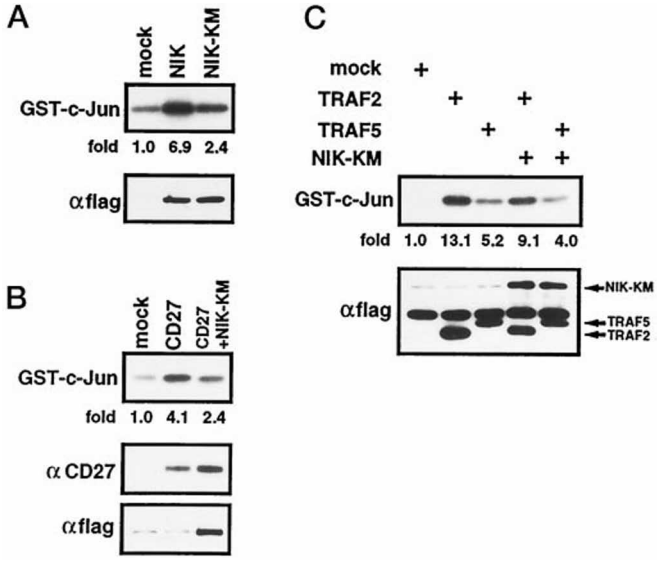

FIG. 6. **Involvement of NIK in SAPK/JNK activation by CD27, TRAF2, and TRAF5.** *A*, activation of SAPK/JNK by NIK. HEK293 cells were co-transfected with 0.5 μg of HA-SAPK and 1.5 μg of Flag-NIK or Flag-NIK-KM. HA-SAPK was immunoprecipitated, and *in vitro* phosphorylation of GST-c-Jun-(1–79) was performed as in Fig. 5. The expression levels of Flag-NIK and Flag-NIK-KM were determined by immunoblotting with anti-Flag mAb. *B*, inhibition of CD27-mediated SAPK/JNK activation by NIK-KM. HEK293 cells were co-transfected with 0.5 μg of HA-SAPK and 1 μg of CD27 along with or without 0.5 μg of Flag-NIK-KM. HA-SAPK was immunoprecipitated, and *in vitro* phosphorylation of GST-c-Jun-(1–79) was performed as in Fig. 5. The expression levels of CD27 and Flag-NIK-KM were determined by immunoblotting with anti-CD27 or anti-Flag mAb. *C*, inhibition of TRAF2- and TRAF5-mediated SAPK/JNK activation by NIK-KM. HEK293 cells were co-transfected with 0.3 μg of HA-SAPK and 1 μg of Flag-TRAF2 or Flag-TRAF5 along with or without 1 μg of Flag-NIK-KM. HA-SAPK was immunoprecipitated, and *in vitro* phosphorylation of GST-c-Jun-(1–79) was performed as in Fig. 5. The expression levels of Flag-TRAF2, Flag-TRAF5, and Flag-NIK-KM were determined by immunoblotting with anti-Flag mAb. The positions of Flag-tagged proteins are indicated at the *right*.

their use of a similar set of TRAF proteins. However, these receptors display unique functions *in vivo* at several levels. For example, CD40 is involved in class switching and proliferation in B cells (35), and CD27 enhances IgG production in B cells (3). CD30 is involved in some types of negative selection in the thymus (36), and LT-βR is implicated in lymph node development (37, 38). The unique functions *in vivo* may result in part from the different tissue expression patterns, in combination with the distinct expression patterns of their cognate ligands, as well as for each TRAF. Indeed, follicular dendritic cells, which play an important role in germinal center formation, exhibit high expression of LT-βR and TRAF5 (39) but little or no detectable TRAF2. The absence of LT-βR on lymphocytes distinguishes this receptor from lymphocyte-specific CD27. Clearly, TRAF2 and TRAF5 have some redundant functions in these tissue culture models; however, other interacting molecules may distinguish the roles of TRAF2 and TRAF5 *in vivo*. For example, TRAF5 does not interact with the TRAF2-binding proteins, TRADD[2] or IAPs (40). On the other hand, TRAF5 interacts with TRAF3, but TRAF2 does not.[2] These results suggested that TRAF2 and TRAF5 could exert unique functions through interaction with distinct sets of downstream signaling molecules. The recent generation of TRAF2-deficient mice revealed that SAPK/JNK activation, but not NF-κB activation, by TNF is abrogated in these mice (41). Our preliminary results showed that a truncated TRAF5 partially inhibited NF-κB activation by TNF, suggesting that TRAF2 and TRAF5 may act redundantly in NF-κB activation by TNF. In contrast, SAPK/JNK activation may be more efficiently mediated by TRAF2 than TRAF5. Consistent with this notion, a higher potential of TRAF2 to activate SAPK/JNK (Fig. 5*B*) and truncated TRAF2 to inhibit CD27-mediated SAPK/JNK activation were noted (Fig. 5*C*). At this moment, it remains to be determined whether TRAF2 and TRAF5 are functionally redundant in signals via CD27 and other members of the TNF-R family in TRAF2- and TRAF5-deficient mice.

Acknowledgments—We thank Masaki Kashiwada, Kyoko Yokota, Hitoshi Kikutani, Toshiki Watanabe, Eisuke Nishida, George Mosilaos, and Elliott Kieff for reagents. We also thank Sachiko Sakon for technical assistance.

[2] H. Akiba and H. Nakano, unpublished data.

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