Reimagine your discoveries
Amnis® ImageStream®X Mk II and FlowSight® Imaging Flow Cytometers
Luminex complexity simplified.
Learn more >

The Journal of Immunology

RESEARCH ARTICLE | NOVEMBER 01 1998

NF-κB Activation in CD27 Signaling: Involvement of TNF Receptor-Associated Factors in Its Signaling and Identification of Functional Region of CD27[1] FREE

Hiroyasu Yamamoto; ... et. al

J Immunol (1998) 161 (9): 4753–4759.
https://doi.org/10.4049/jimmunol.161.9.4753

Related Content

Inhibition of Terminal Differentiation of B Cells Mediated by CD27 and CD40 Involves Signaling through JNK

J Immunol (December,2010)

Agonist Anti-Human CD27 Monoclonal Antibody Induces T Cell Activation and Tumor Immunity in Human CD27–Transgenic Mice

J Immunol (October,2013)

Therapeutic efficacy of a fully human anti-CD27 monoclonal antibody in a murine colon carcinoma model (162.23)

J Immunol (May,2012)

Downloaded from http://journals.aai.org/jimmunol/article-pdf/161/9/4753/1094561/im2198004753o.pdf by guest on 01 June 2023

NF-κB Activation in CD27 Signaling: Involvement of TNF Receptor-Associated Factors in Its Signaling and Identification of Functional Region of CD27[1]

Hiroyasu Yamamoto, Tadamitsu Kishimoto,[2] and Seijiro Minamoto

CD27 belongs to TNF receptor family, and it is unique in this family for its disulfide-linked homodimerization of 55-kDa monomers. In the present study we demonstrate that overexpression of CD27 in 293 cells induces a low level of NF-κB activation, and the ligation of the receptor by its corresponding ligand (CD70) augments this signal dramatically. Either TNF receptor-associated factor-2 (TRAF2) or TRAF3 binds to the CD27 molecule from the coimmunoprecipitation experiment. This NF-κB activation signal is inhibited by dominant negative TRAF2 or intact TRAF3, indicating that TRAF2 and TRAF3 works as a mediator and an inhibitor, respectively. The activated NF-κB complex contains at least two components, p50 and p65, but not p52. All these phenomena have also been observed in the TNF receptor type II, CD30 and CD40 signaling system, indicating that this receptor family uses the common or similar molecules for this signal. Finally, we identified the 13-amino acid alignment in the cytoplasmic region of the CD27 molecule (residues 238–250 amino acids), which is critical for the NF-κB activation signal and also for its association with TRAFs. This amino acid alignment contains the EEEG sequence, which is essential for interaction of CD30 or CD40 with TRAFs (TRAF1 and TRAF2, but not TRAF3), and also contains the PIQED sequence, which is similar to PXQXT that is known to be necessary for interaction of TNF receptor II and CD30 with TRAFs (TRAF1, 2, and 3). ***The Journal of Immunology,*** **1998, 161: 4753–4759.**

Downloaded from http://journals.aai.org/jimmunol/article-pdf/161/9/4753/1094561/im2198004753o.pdf by guest on 01 June 2023

The CD27 Ag is a 120-kDa type I transmembrane phosphoglycoprotein composed of disulfide-linked 55-kDa monomers (1–4) and belongs to the TNF receptor family, including TNF receptor (TNFR I and TNFR II), low affinity nerve growth factor receptor ($p75^{LNGFR}$), CD40, CD30, CD95 (Fas/Apo I), 4–1BB, and OX40 (5–19). CD27 is expressed on medullary thymocytes (1, 20), on the surface of 75% of human peripheral blood T cells (equally distributed between $CD4^+$ and $CD8^+$ subsets) (1, 2), on a subpopulation of B cells (21, 22), and on NK cells (23). The activation of human T cells via the TCR-CD3 complex leads to expression of CD27, which peaks after 3 to 4 days and then gradually decreases after prolonged activation (24). CD70, a ligand for CD27, is a type II transmembrane glycoprotein belonging to the TNF family and is expressed by activated T and B cells (25–27). CD27/CD70 interaction augments the induction of T cell activation (28), B cell activation (IgG and IgM production) (29, 30), and NK cell killing activity (31).

The TNF receptor-associated factors (TRAFs)[3] are composed of at least six members (TRAF1–6) (32–39). TRAF1 and TRAF2 were found to associate with the cytoplasmic domain of TNFR II as a heterodimer (32). TRAF3 was identified as a molecule binding to cytoplasmic domain of CD40 (33–35), but was subsequently known to associate with the lymphotoxin-β receptor, TNFR II, and CD30. TRAF4 (CART1) was cloned from a breast carcinoma cell line, and this is the only TRAF that has been localized to the nucleus and may not be associated with a surface receptor (36). TRAF5 was shown to bind to the lymphotoxin-β receptor (37, 38). TRAF6 was identified as a mediator of the IL-1 signal (39). TRAF proteins share a conserved approximately 230-amino acid C-terminal TRAF domain (TRAF-N and TRAF-C) and also N-terminal ring finger and zinc fingers (except TRAF1). The TRAF-C domain is responsible for oligomerization and association to each molecule of the TNF receptor family. The TRAF-N domains of TRAF1 and TRAF2 have been shown to mediate the binding to members of the family of inhibitors of apoptosis proteins (40). Functionally, TRAF2 is a common signal mediator for TNFR II, CD40, and CD30, which lead to activation of the transcription factor, NF-κB (41, 42), while TRAF3 is a common signal inhibitor for them. It was recently reported that TRAF2 binds TRADD (the TNFR I-associated death domain-containing protein) (43) and mediates the activation of NF-κB by TNFR I (44). Malinin et al. have cloned NIK (NF-κB-inducing kinase) (45, 46), which is similar to mitogen-activated protein kinase kinase kinase and binds to TRAF2 and I-κB kinase-1 and -2 (IKK-1 and -2). These three kinases form a complex, and this complex phosphorylates I-κBα and I-κBβ, followed by their degradation (47–49).

In the current study, we demonstrated that CD27 signal activates NF-κB activity and suggested that this signal may be mediated by TRAF2, as the other member of TNFR family did, although CD27 is a unique member in that it forms disulfide-linked homodimer. In addition, TRAF3 displayed an inhibitory effect on this signal. This activated NF-κB complex contained at least two components, p50 and p65. Finally, we determined the essential cytoplasmic 13-amino acid alignment (residues 238–250 amino acids) of the CD27 molecule that is needed not only for the NF-κB-activating

Department of Medicine III, Osaka University Medical School, Osaka, Japan

Received for publication November 14, 1997. Accepted for publication June 23, 1998.

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.

[1] This work was supported by a Grant-in-Aid from the Ministry of Education, Science and Culture, Japan.

[2] Address correspondence and reprint requests to Dr. Tadamitsu Kishimoto, 2-2 Yamada-oka, Suita-City, Osaka 565-0871, Japan. E-mail address: kishimot@imed3.med.osaka-u.ac.jp

[3] Abbreviations used in this paper: TRAF, TNF receptor-associated factor; NIK, nuclear factor-κB-inducing kinase; IKK, I-κB kinase; hTRAF, human TRAF; mTRAF, murine TRAF; dnTRAF, dominant negative TRAF; EMSA, electrophoretic mobility shift assay; wt, wild type; ECL, enhanced chemiluminescence.

Copyright © 1998 by The American Association of Immunologists

0022-1767/98/$02.00

signal but also for its interaction with TRAFs. This 13-amino acid alignment contained the EEEG sequence, which is critical to CD30 or CD40 for their association with TRAF1 or TRAF2 (50, 51), and the PIQED sequence, which is similar to PXQXT and is critical to TNFR II or CD30 for their interaction with TRAF2 and TRAF3.

Materials and Methods

Cells

293 cells were maintained in DMEM with 10% (v/v) FCS.

Antibodies

Rabbit polyclonal anti-NF-κB p65 Ab (0.1 mg/ml; sc-109), rabbit polyclonal anti-NF-κB p50 Ab (0.1 mg/ml; sc-114), rabbit polyclonal anti-NF-κB p52 Ab (0.1 mg/ml; sc-297; Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-human CD27 Ab (M-T271; Ancell Technology Research Products, Bayport, MN), mouse monoclonal anti-human CD70 Ab (HNE51; Immunotech, Luminy, France), mouse IgG1 (BioPur, Bubendorf, Switzerland), mouse anti-c-Myc mAb (OP-10; Oncogene, Cambridge MA), and horseradish peroxidase-coupled goat anti-rabbit IgG(H+L) (Zymed, South San Francisco, CA) were purchased.

DNA construction

DNA fragments encoding human CD27 (hCD27), human CD70 (hCD70), human TRAF1 (hTRAF1), and human TRAF3 (hTRAF3) were amplified by PCR from the human cDNA library prepared from activated mono-nuclear cell mRNA with the following specific primers: 5′hCD27 (5′-CAGCAAGCT TAACTGGGCACAGAAAGGAGCCG-3′), 3′hCD27 (5′-AGGTTCTA GATCAGGGGGAGCAGGCAGGC-3′), 5′hCD70 (5′-GCTCTCTAGACC CATCGCCCCTCCTGCGCTA-3′), 3′hCD70 (5′-TCAGTCTAGATGGTC AGGGGCGCACCCACTG-3′), 5′hTRAF1 (5′CCCTGAATTCGCCTC CAGCTCAGGCAGCAGTC3′), 3′hTRAF1 (5′-CCCGGGATCCCTAAGT GCTGGTCTCCACAATG-3′), 5′hTRAF3 (5′-TCCTGAATTCGAGTCGA GTAAAAAGATGGACT-3′), and 3′hTRAF3 (5′-CCTCGGATCCTA CTTATCAGGGATCGGGCAGA-3′). DNA fragment encoding murine TRAF2 (mTRAF2) was amplified by PCR from a murine splenic cDNA library using the following primers: 5′mTRAF2 (5′-GTAAGGATCCTG GCTGGCTGCAGCCAGTGTGACTTC-3′) and 3′mTRAF2 (5′-GCA GGGATCCCTAGAGTCCTGTTAGGTCCACA-3′). The DNA fragment encoding dominant negative TRAF2 (dnTRAF2) (42), which lacks the N-terminal 86 amino acids of TRAF2 was also amplified by PCR from TRAF2 cDNA using primers 5′dnTRAF2 (5′-GGAAGAATTCC AGTCTATAAGCCCAGGAAGCC-3′) and 3′dnTRAF2 (5′-CCCGGGATC CCTAAGTGCTGGTCTCCACAATG-3′). For expression in mammalian cells, TRAFs were inserted into pCDM8 (52) with epitope tag (Myc) constructs at N-terminal. The epitope tag sequence for Myc was MEQKLI SEEDLN. We designated the expression vector for the Myc-TRAF fusion sequence as pCDM-Myc-TRAFs. The cDNA for hCD27 was cloned into the pCDM8 vector. Subsequently, three C-terminus-truncated hCD27 mutants lacking 10, 23, or 41 amino acids (see Fig. 6*A*) were generated by PCR. The cDNA for hCD70 was also cloned into the pEFBOS vector. All constructs were confirmed by partial sequencing.

p-55IgκLuc (53) containing three tandemly repeated κB motifs upstream of luciferase structural gene was a gift from Dr. T. Fujita (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). pCH110 (Pharmacia Biotech, Uppsala, Sweden) expressing the β-galactosidase gene was used to normalize the luciferase activity.

DNA transfection

Basically, 293 cells (1×10^6) were seeded on a 60-mm collagen-coated dish and transfected the next day with 6.5 μg of plasmid DNA/dish by the calcium phosphate method. After 4-h incubation with the DNA-calcium phosphate precipitates, the culture medium was changed to fresh medium. Electrophoretic mobility shift assays (EMSAs) and luciferase assays were performed 48 to 72 h after initiation of the transfection.

Nuclear extracts preparation and EMSAs

Nuclear extract preparation was performed with the modified Schreiber's method (54). Cells were washed twice with PBS and resuspended in 400 μl of ice-cold lysis buffer containing 10 mM HEPES-KOH (pH 7.8), 10 mM KCl, 0.1 mM EDTA (pH 8.0), 1 mM DTT, and antiprotease mixture (0.5 mM PMSF and 2 μg/ml each of aprotinin, leupeptin, and pepstatin A; final concentrations). Following a 15-min incubation on ice, 25 μl of 10% Nonidet P-40 was added, and then cells were briefly vortexed and centrifuged at 5000 × *g* for 1 min at 4°C. The supernatants (nonnuclear fractions) were discarded, and the nuclear pellets were resuspended in 100 μl of ice-cold nuclear extraction buffer containing 50 mM HEPES-KOH (pH 7.8), 420 mM KCl, 0.1 mM EDTA (pH 8.0), 5 mM $MgCl_2$, 20% (v/v) glycerol, 1 mM DTT, and the antiprotease mixture; vortex briefly; and vigorously shaken on a shaking platform at 4°C for 15 min. After that, the extracted nuclear fractions were centrifuged for 15 min (15,000 × *g*, 4°C), and the resulting supernatants (the nuclear extracts) were aliquoted and stored at −70°C before use. Aliquots of the nuclear extracts were processed for protein content determination using Bradford reagent (Bio-Rad, Herculus, CA). Double-stranded oligonucleotides (5′-AGTTGAGGGGACTTTC CCAGGC-3′) containing NF-κB binding sites were ^{32}P labeled using polynucleotide kinase (Toyobo, Tokyo, Japan). Binding reactions were performed in 16 μl of binding buffer containing 25 mM HEPES-KOH (pH 7.8), 0.5 mM EDTA-NaOH (pH 8.0), 50 mM KCl, 10% (v/v) glycerol, 0.5 mM PMSF, 0.5 mM DTT, and 2 μg of double-stranded poly(dI-dC). Two micrograms of nuclear extract was then added to the binding mixture and allowed to equilibrate for 15 min at room temperature. Alternatively, for competition or supershift experiments, the above binding reactions were conducted in the presence of a 100-fold excess of cold competitors or 0.3 μg of anti-polyclonal Abs raised against members of the NF-κB/Rel family. Finally, $>10^5$ cpm of ^{32}P-labeled double-stranded oligonucleotides containing the consensus NF-κB sequence were added to the binding mixtures and incubated for 30 min at room temperature. The resulting samples were separated on 4% polyacrylamide gels. After electrophoresis, gels were subsequently dried and exposed to Fuji medical x-ray film (Tokyo, Japan) at −80°C with intensifying screens.

Flow cytometry

After 2-day incubation of the transfection of 293 cells, the cells were immunostained with anti-human CD27 Ab (M-T271) or anti-human CD70 Ab (HNE51) and a FITC-conjugated goat anti-mouse IgG and analyzed by FACScan (Becton Dickinson, San Jose, CA) for CD27 or CD70 expression, respectively.

Luciferase activity assays

Luciferase activity assays were performed using the luciferase assay system (Promega, Madison, WI). Luminescence was quantitated by a chemiluminescence counter (Lumat LB9501, Berthold, Nashua, NH). β-Galactosidase activity was also measured using the β-galactosidase enzyme assay system (Promega).

Coimmunoprecipitation and Western blot analysis

293 cells (5×10^6 cells) were transfected with each pCDM8-Myc-TRAF together with pCDM8-hCD27 (wild type (wt)) or hCD27 deletion mutant by the calcium phosphate method. Transfected cells were harvested 48 h after transfection. Cells were then washed with PBS and lysed in 400 μl of ice-cold 1% Nonidet P-40 lysis buffer containing 50 mM HEPES (pH 7.55), 150 mM NaCl, 0.4 mM EDTA, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, 10 μg/ml aprotinin, and 100 μg/ml PMSF. Sixty microliters of each cell lysate (cell membrane and cytoplasm fractions) was mixed with 20 μl of 4× sample buffer (containing 0.0625 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 0.001% bromphenol blue), and 10 μl of each sample was used in SDS-PAGE analysis. One hundred and fifty microliters of each cell lysate was incubated with 1 μg of murine anti-human CD27 Ab (M-T271) or the same amount of isotype-matched murine Ig (control IgG) and 40 μl of protein G. The beads were washed four times with lysis buffer, and bound proteins were resuspended in 40 μl of 2× sample buffer. Ten microliters of each sample was fractioned by 4 to 20% gradient SDS-PAGE and transferred to an Immobilon membrane (Millipore, Bedford, MA). Western blot analysis to detect the Myc epitope was performed with anti-Myc Ab (from rabbit) and visualized with horseradish peroxidase-coupled goat anti-rabbit IgG(H+L) using the enhanced chemiluminescence (ECL) system according to the protocol of the manufacturer (Amersham, Aylesbury, U.K.).

Results

NF-κB activation by overexpression of CD27 or both CD27 and CD70 (CD27 ligand) in 293 cells

Some members of the TNFR family have been reported to activate the nuclear transcriptional factor, NF-κB. Therefore, we examined whether CD27 could transduce the signal for NF-κB activation by monitoring the luciferase activities in 293 cells. 293 cells were

Downloaded from http://journals.aai.org/jimmunol/article-pdf/161/9/4753/1094561/im2198004753o.pdf by guest on 01 June 2023

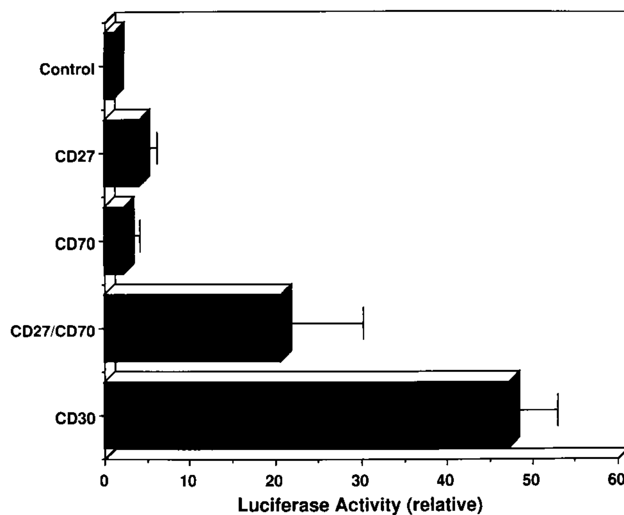

FIGURE 1. NF-κB activation triggered by overexpression of CD27 alone or together with CD70 in 293 cells. 293 cells (1 × 10^6) were transiently transfected with pCDM8 vector alone, 1 μg of pCDM8-hCD27, or 1 μg of pCDM8-hCD27 together with 1 μg of pEFBOS-hCD70 in addition to 1 μg of p-55IgκLuc vector (NF-κB reporter plasmid) and 0.5 μg of pCH110 (β-galactosidase expression vector) by the calcium phosphate method. In all the transfection experiments, total amounts of plasmid were kept constant by adding pCDM8 vector. After 48 to 72 h, luciferase activities were determined and normalized on the basis of β-galactosidase expression. The relative luciferase activity represents the percentage of normalized luciferase activities relative to that obtained from the transfection with pCDM8-hCD27. Error bars represent the SE from triplicate transfection trials.

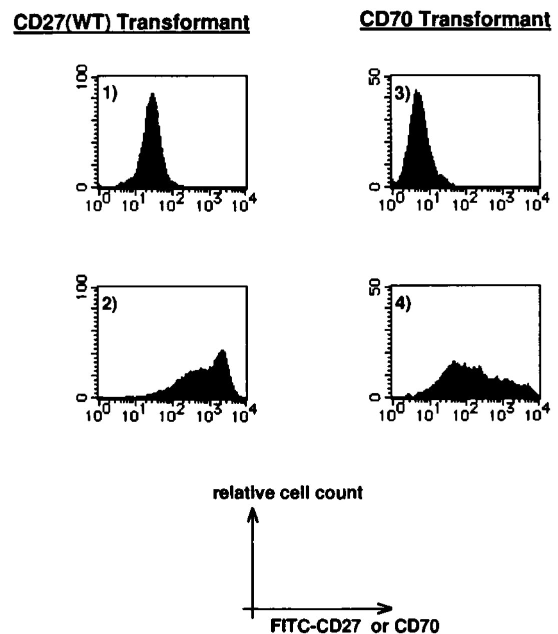

FIGURE 2. Cell surface expression of CD27 or CD70 on 293 transformants. Cell surface expression of hCD27 or hCD70 on 293 transfectants was assessed by indirect immunofluorescence using 1 and 2) anti-CD27 mAb (M-T271), or 3 and 4) anti-CD70 mAb (HNE51) and FITC-conjugated goat anti-mouse IgG. Analyses were performed with logarithmic scales on the *x*-axis (fluorescence). 1) pCDM8-unrelated molecule alone; 2) pCDM8-hCD27; 3) pEFBOS-unrelated molecule alone; 4) pEFBOS-hCD70 transformant.

transiently transfected with the CD27 expression vector (pCDM8-hCD27) or pCDM8 with no insert as a control vector. In pCDM8-hCD27-transfected 293 cells, NF-κB activation was only 2 to 5 times higher than that in control cells. However, when we cotransfected pCDM8-hCD27 together with pEFBOS-hCD70 in 293 cells, the activity was up-regulated to a level about 20 times higher than the control value (Fig. 1). In pEFBOS-hCD70-transfected 293 cells, no NF-κB activation was found. These results indicate that expression of CD27 alone is not sufficient for the full level of NF-κB activation in 293 cells, and additional CD70 stimulation augmented this signal, although the activation produced by the CD27/CD70 system is still lower than that produced by the CD30 signaling system. Expression profiles of hCD27 and hCD70 molecules in transient transformants were demonstrated in Figure 2.

EMSAs

To determine the involving NF-κB components, we performed EMSAs with the ^{32}P-labeled double-stranded NF-κB oligonucleotides (Fig. 3*A*). EMSAs showed that CD70 did not activate NF-κB, CD27 alone did, and CD27 together with CD70 induced the most activity. These results are identical with that obtained by the luciferase activity assay system. In addition, each Ab to NF-κB subunit p65 or p50 caused induction of a supershift of the protein-NF-κB probe complex, but anti-p52 Ab had no effect (Fig. 3*B*). These results indicate that the signal from CD27 can activate the NF-κB complex, which contains at least two components, p65 and p50, in 293 cells.

Interaction between TRAFs and the CD27 molecule

In the NF-κB activation signal of the TNFR II, CD40, and CD30 systems, either TRAF2 or TRAF3 works as an attaching molecule to the cytoplasmic region of the receptors and plays an important role in their signals (32–35, 41, 42). We tried to determine whether the cytoplasmic domain of CD27 would bind to TRAFs by a yeast two-hybrid system. However, any obvious interactions between the cytoplasmic domain of CD27 and each TRAF (TRAF1–3)

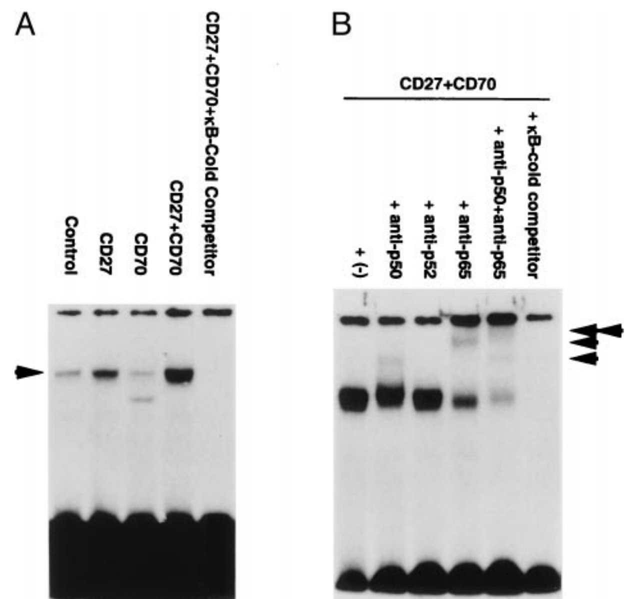

FIGURE 3. Identification of NF-κB components activated by CD27 alone or together with CD70. 293 cells were transfected with pCDM8 vector alone or the expression vector for CD27 (wt) (pCDM8-hCD27(wt)) and CD70 (pEFBOS-hCD70). Two days later, the cells were used for EMSAs. In *A*, 2-μg aliquots of the nuclear extracts were incubated with ^{32}P-labeled double-stranded NF-κB oligonucleotides. Arrows indicate the κB double-stranded NF-κB oligonucleotide complexes. In *B*, the reaction mixture was incubated with anti-p50, anti-p65, anti-p52, or unlabeled competitor oligonucleotides. The arrows show the supershifted band. All EMSAs were performed as described in *Materials and Methods*.

Downloaded from http://journals.aai.org/jimmunol/article-pdf/161/9/4753/1094561/im2198004753o.pdf by guest on 01 June 2023

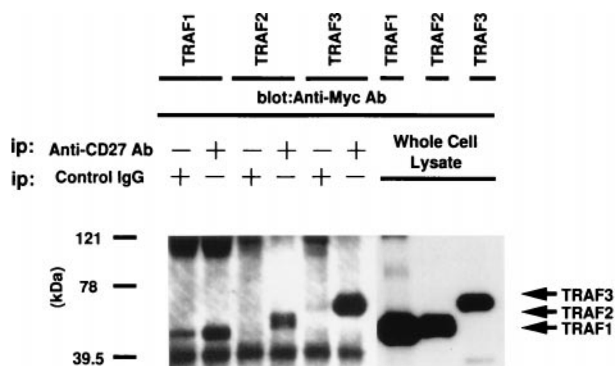

FIGURE 4. Interaction between TRAFs and the CD27 molecule. 293 cells were transiently transfected with each pCDM8-Myc-TRAFs (TRAF1-TRAF3) and pCDM8-hCD27 (wt). After 48 h, each cell lysate was subjected to immunoprecipitation with control mIgG1 or mouse anti-hCD27 Ab. Each coimmunoprecipitated Myc-TRAF was fractionated on SDS-PAGE and analyzed by Western blot with anti-Myc Ab followed by ECL. Arrows indicate coimmunoprecipitated Myc-TRAFs molecules.

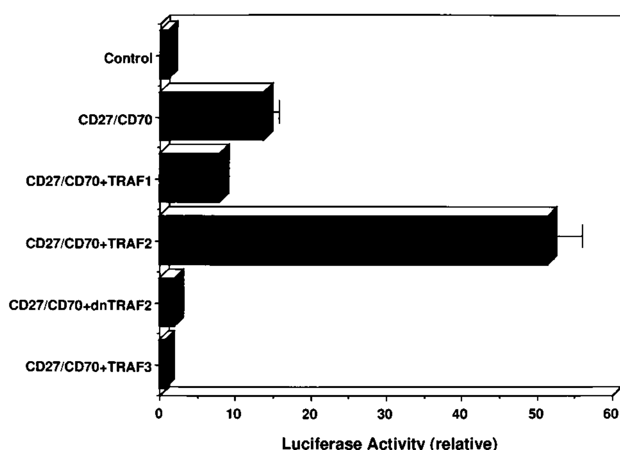

FIGURE 5. Involvement of TRAFs in CD27-induced NF-κB activation signal. pCDM8-hCD27 and pEFBOS-hCD70 (0.3 μg each) were cotransfected into 293 cells with 3.4 μg of pCDM8 with no insert or with pCDM8 expressing TRAF1, TRAF2, TRAF3 or dnTRAF2 (pCDM8-Myc-TRAF2) in addition to 0.5 μg of pCH110 and 1 μg of p-55IgκLuc vector by the calcium phosphate method. The luciferase and β-galactosidase assays were performed as described in *Materials and Methods*. Error bars represent the SE from triplicate transfection trials.

were not observed (data not shown). We speculated that the dimerization of CD27 may be necessary for the interaction of CD27 with TRAFs, because CD27 is a unique molecule among the TNF receptor family in that it is expressed in a homodimerized form. Therefore, we investigated the interactions between whole human CD27 and TRAFs by coimmunoprecipitation experiments. pCDM8-hCD27 and each pCDM8-Myc-TRAF were cotransfected in 293 cells, and coimmunoprecipitation of TRAFs with the CD27 molecule was demonstrated by anti-Myc immunoblot. The interaction between either TRAF2 or TRAF3 and the CD27 molecule was observed (Fig. 4). As for TRAF1, the possibility of association with the CD27 molecule was suspected, but the clear interaction was not detected because of its nonspecific background (Figs. 4 and 8). These results indicate that at least TRAF2 and TRAF3 can interact with the cytoplasmic region of the CD27 molecule and raise the possibility that CD27 might use TRAFs as effectors for NF-κB activation.

Involvement of TRAFs in NF-κB activation induced by CD27/CD70 interaction

To elucidate the involvement of TRAFs in NF-κB activation in CD27 signaling, we cotransfected each pCDM8-TRAF with both pCDM8-hCD27 (wt) and pEFBOS-hCD70 and performed reporter assays, because the interaction between TRAFs and the CD27 molecule was shown in a coimmunoprecipitation experiment. From the data of the luciferase activity assays, intact TRAF2 augmented the NF-κB activation (Fig. 5). This result suggests that TRAF2 is a mediator of the signal induced by CD27/CD70 interaction. However, this result must be interpreted with caution, because overexpression of TRAF2 alone also induces NF-κB activation (Ref. 42, our data, and data not shown). Therefore, we constructed pCDM8-dnTRAF2. As we expected, dnTRAF2 clearly blocked the NF-κB activation triggered by CD27/CD70 interaction (Fig. 5). These two experiments indicate that the CD27 signal for NF-κB activation should be transmitted through at least TRAF2. TRAF1 also reduced this NF-κB activation, although the reduction level was partial. In contrast to TRAF2, TRAF3 blocked NF-κB activation completely, suggesting that TRAF3 works as a negative regulator in CD27 signaling, as demonstrated previously in TNFR II, CD40, and CD30 signaling.

Critical cytoplasmic region of CD27 in NF-κB activation signal

To identify the cytoplasmic region of CD27 necessary for NF-κB activation, we constructed the expression vectors for the three kinds of CD27 mutants, lacking 10, 23, or 41 amino acids at the carboxyl-terminus (Fig. 6*A*). All the mutations were confirmed by partial DNA sequencing, and the expression profiles of each mutant CD27 molecule and CD70 molecule in transiently cotransfected 293 cells were demonstrated in Figure 6*C*. On each transformant, wild-type or mutant CD27 molecules and CD70 molecules were expressed. Then in cotransfected 293 cells with either pCDM8-hCD27 (wt) or one of the hCD27 deletion mutants and pEFBOS-hCD70, we measured NF-κB activities by monitoring luciferase activities. Deletion of 10 carboxyl-terminal amino acids did not affect NF-κB activation, but the mutant lacking 23 or 41 amino acids failed to activate NF-κB completely (Fig. 7). These results show that a 13-amino acid alignment (residues 238–250 amino acids) is critical for NF-κB activation by CD27/CD70 interaction. Subsequently, using CD27 (wt) or these CD27 deletion mutants, their interaction with each TRAF was examined by coimmunoprecipitation and Western blot analyses. In accordance with the results of luciferase assays, the interaction between TRAFs (TRAF2 and 3) and the CD27 (wt) molecule or the CD27 mutant lacking 10 carboxyl-terminal amino acids was detected; however, the obvious association of TRAFs (TRAF2 and 3) with the CD27 mutant lacking 23 amino acids was not observed (Fig. 8). Again, the high background of TRAF1 was observed (Fig. 8), and we failed to make it clear whether TRAF1 could bind to the CD27 molecule. From these results, the 13-amino acid alignment (residues 238–250 amino acids) is also critical for interaction with TRAFs as well as activation of NF-κB signaling.

Discussion

CD27 is a member of TNFR family that includes two groups. One group has a death domain motif in its own receptor, and its activation induces apoptosis, and the other group induces NF-κB activation as an intracellular signal. Several reports suggested that CD27 belongs to the latter group by showing that CD27/CD70 interaction activates T, B, and NK cells, and CD27 does not have a death domain (28–31). In this study we directly demonstrate that CD27/CD70 interaction induces NF-κB activation. Overexpression of CD27 molecules alone activates NF-κB signal in 293 cells,

Downloaded from http://journals.aai.org/jimmunol/article-pdf/161/9/4753/1094561/im2198004753o.pdf by guest on 01 June 2023

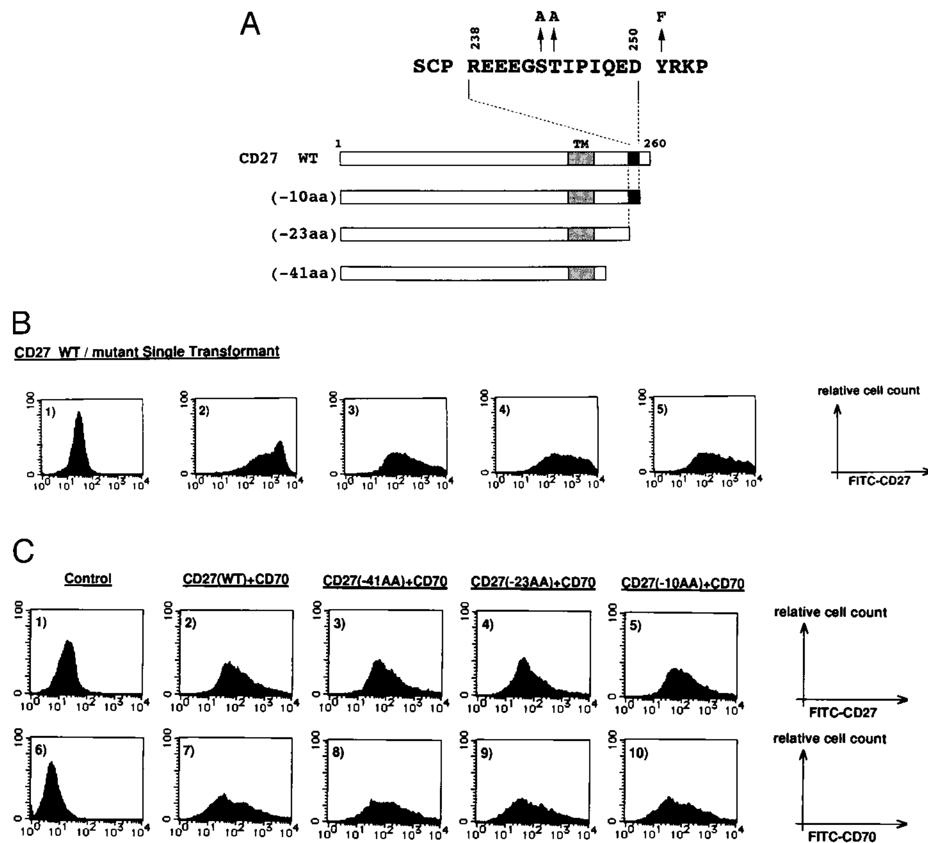

FIGURE 6. Expression of each CD27 mutant and CD70 molecule on cotransfected 293 cells. *A*, Schematic diagram of CD27 mutants. Three kinds of C-terminus-truncated CD27 mutants were made by PCR (CD27 (−41 amino acids), CD27 (−23 amino acids), and CD27 (−10 amino acids)). *B*, Cell surface expression of CD27 (wt) or each mutant was assessed by indirect immunofluorescence using mouse anti-hCD27 Ab (M-T271) and FITC-conjugated goat anti-mouse IgG. Analyses were performed with logarithmic scales on the *x*-axis (fluorescence). 1) pCDM8-unrelated molecule, 2) pCDM8-hCD27 (wt), 3) pCDM8-hCD27 (−41 amino acids), 4) pCDM8-hCD27 (−23 amino acids), or 5) pCDM8-hCD27 (−10 amino acids) was solely transfected. *C*, Cell surface expression of each hCD27 mutant or hCD70 on 293 transfectants was assessed by indirect immunofluorescence using anti-CD27 Ab (no. 1–5) or anti-CD70 Ab (no. 6–10) and FITC-conjugated goat anti-mouse IgG. Analyses were performed with logarithmic scales on the *x*-axis (fluorescence). pEFBOS-hCD70 was cotransfected with pCDM8-hCD27 (wt; no. 2 and 7), pCDM8-hCD27 (−41 amino acids; no. 3 and 8), pCDM8-hCD27 (−23 amino acids; no. 4 and 9), or pCDM8-hCD27 (−10 amino acids; no. 5 and 10). 1) pCDM8-unrelated molecule or 6) pEFBOS-unrelated molecule was solely transfected.

but the induction level is weaker than that of any other of this group's molecules such as CD30 or CD40. Coexpression of CD70 together with CD27 remarkably augments this signal; therefore, CD70 stimulation is needed for sufficient NF-κB activation signal in 293 cells.

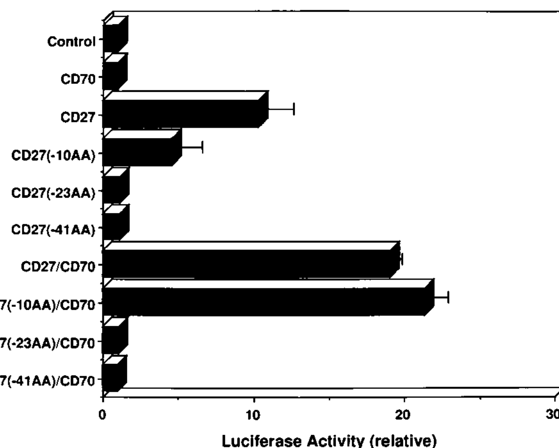

FIGURE 7. Determination of the critical region of CD27 in NF-κB activation signal. One microgram of pEFBOS-hCD70 and each 1 μg of pCDM8-hCD27 mutant were cotransfected with 0.5 μg of pCH110 and 1 μg of p-55IgκLuc vector by the calcium phosphate method. The luciferase and β-galactosidase assays were performed as described in *Materials and Methods*. Error bars represent the SE from triplicate transfection trials.

In the cascade of NF-κB activation by CD27, we suggest that TRAF2 may mediate this signal, and TRAF3 has an inhibitory effect on this signal, because either dominant negative TRAF2 or intact TRAF3 inhibits the CD27-mediated NF-κB action. This phenomenon was also seen in TNFR type II-, CD30-, and CD40-mediated signals. Interaction between TRAFs and these TNFR family members was demonstrated in several reports, by the yeast two-hybrid system, and by coimmunoprecipitation and Western blot analyses using mammalian cells. First, we performed the yeast two-hybrid assays, but we could not observe any interaction between the cytoplasmic region of CD27 and TRAFs. Because CD27 exists as a disulfide-bonded homodimer, we supposed that the yeast two-hybrid system could not work well. For this reason, we performed a coimmunoprecipitation experiment using mouse anti-human CD27 Ab (M-T271) and 293 cells transiently expressing whole CD27 molecule and either of the TRAFs. As a result, we observed the association between CD27 and TRAF2 or TRAF3. It is not clear why we could not detect the interaction between CD27 and TRAFs in the yeast two-hybrid system, but we think it is plausible that the dimerized form of the CD27 molecule might be necessary for the interaction between CD27 and each TRAF molecule. Additional experiments might be needed to prove our suggestion.

Downloaded from http://journals.aai.org/jimmunol/article-pdf/161/9/4753/1094561/im2198004753o.pdf by guest on 01 June 2023

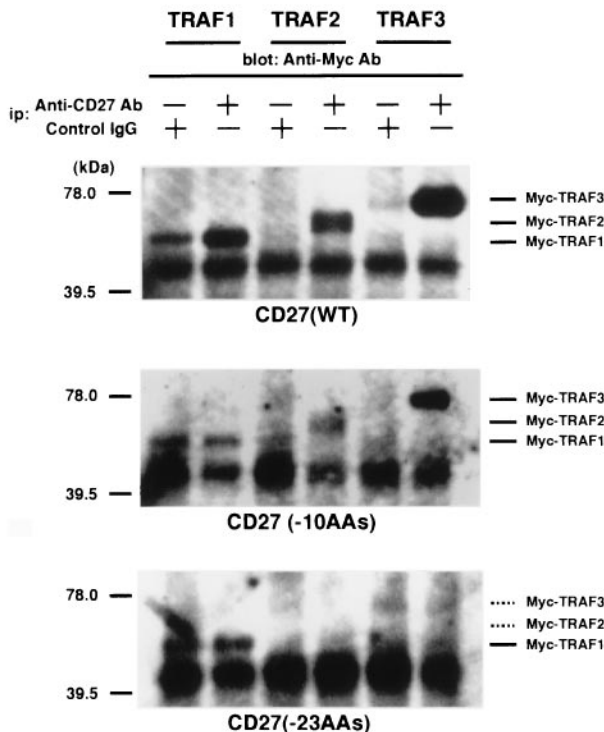

FIGURE 8. Critical region of the CD27 molecule for interaction with TRAFs. 293 cells were transiently transfected with each pCDM8-Myc-TRAFs (TRAF1-TRAF3) and pCDM8-hCD27 (wt), hCD27 (−10 amino acids), or hCD27 (−23 amino acids). After 48 h, each cell lysate was subjected to immunoprecipitation with control mIgG1 or mouse anti-hCD27 Ab. Each coimmunoprecipitated Myc-TRAF was separated on SDS-PAGE and analyzed by Western blot with anti-Myc Ab followed by ECL. Arrows indicate coimmunoprecipitated Myc-TRAFs molecules.

With the truncated forms of CD27, we determined the critical region involved in NF-κB activation. From the data of single transformants, CD27 (−23 amino acids) and CD27 (−41 amino acids) could not activate NF-κB completely, but CD27 (−10 amino acids) could activate it, although the activation level is lower than that of intact CD27. This might be caused by the slight decrease in CD27 expression (as shown in Fig. 6*B*) and/or by the reduced affinity for TRAFs due to subtle conformational change by deletion of carboxyl-terminal 10 amino acids. From the reporter assays using the double transformant of CD27 mutant and CD70, the deletion of 10 carboxyl-terminal amino acids did not affect the CD27 signal, but the deletion of 23 amino acids almost completely abrogated the signal, indicating that this 13-amino acid alignment (residues 238–250 amino acids) is essential for the signal. From coimmunoprecipitation and Western blot analyses, this alignment is also necessary for interaction with TRAFs as well as NF-κB activation. There have been several reports demonstrating the essential domains of TNFR family in its signaling cascades (34, 50, 51, 55, 56). As for CD30, two functional domains exist at the C-terminus (50, 51): element 2A encompasses the sequence PEQET and binds to TRAF1, TRAF2, and TRAF3; element 2B, which is located distal of element 2A, encompasses the sequence EEEGKE and binds to TRAF1 and TRAF2, but not to TRAF3. CD40 contains the EEEGKE motif, and TNFR II contains the PVQET motif. The 13 amino acids critical for CD27 signaling include the EEEG motif, similar to EEEGKE, and there is also a PIQED sequence, similar to PEQET or PVQET at the distal region of EEEG motif. We show at least TRAF2 and TRAF3 can bind to this 13-amino acid sequence of CD27; therefore, it is supposed that TRAF2 may bind to CD27 at both motifs, and TRAF3 may bind at the PIQED motif, although the PIQED sequence is slightly different from the PEQET motif (also recognized as PXQXT). Single amino acid point mutation experiments have been reported as for the interaction between TRAFs and the CD30 molecule (57). From their reports, each single amino acid substitution of this PXQXT motif blocks their interaction, and other amino acid mutants that contain mutations around the intact PXQXT motif also partially or completely inhibit the interaction. Therefore, PXQXT is the core sequence, but the overall conformation including this core sequence is thought to be important for the association.

However, in the case of CD40, Thr^{234} (in the PVQET motif) has been shown to be important in the growth arrest signal in the murine thymoma cell line EL4 and the murine B lymphoma cell line M12 (55), suggesting that the phosphorylation by the TNFR family or some protein kinases is necessary for the interaction of TRAFs through the PXQXT motif, although there is no other suggestive evidence for their possibility. In CD27, the PIQED motif, similar to PXQXT, does not contain any amino acid residue that can be targeted by protein kinase. Therefore, we generated some mutants of CD27 molecules with point mutation at serine, threonine, or tyrosine residue around the critical region and examined the effect on NF-κB activity. We performed the reporter assays using cotransformants with mutant CD27 molecule and CD70 (wt), and we observed that in each transformant (Ser^{243}→Ala, Thr^{244}→Ala, or Tyr^{251}→Phe; Fig. 6*A*), NF-κB activity slightly decreased compared with CD27 (wt), but not significantly (data not shown). This result indicates that at least these three mutated amino acids may not be essential for NF-κB activation.

In the CD27 signaling cascade, not as much information has been reported to date. Kobata et al. (58) demonstrated that CD27 ligation induces calcium mobilization, protein kinase C activation, and protein tyrosine kinase activation that causes tyrosine phosphorylation of cellular substrates, including ZAP-70. Here, we demonstrated that CD27 signaling cascade contains at least the TRAF2-NF-κB activation pathway. Recent reports indicated that TRAF2 activates NIK, and NIK, in turn, activates IKK-1 and IKK-2; these three kinases form a complex, resulting in the phosphorylation of I-κB and its degradation, which leads to NF-κB activation. At present, it is unlikely that the activation of TRAF-NF-κB activation cascade induces calcium mobilization, protein kinase C activation, or protein tyrosine kinase activation. Therefore, CD27 may also activate multiple signaling pathways as observed in tyrosine kinase receptors and cytokine receptors.

Acknowledgments

We thank Dr. T. Fujita (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) for generously providing us the vector p-55IgκLuc, and Drs. Y. Katada and T. Tatekawa (Department of Medicine III, Osaka University Medical School, Osaka, Japan) for technical support of FACS analysis.

References

1. van Lier, R. A. W., J. Borst, T. M. Vroom, H. Klein, P. van Mourik, W. P. Zeijlemaker, and C. J. M. Melief. 1987. Tissue distribution and biochemical functional properties of Tp55(CD27), a novel T cell differentiation antigen. *J. Immunol. 139:1589.*
2. Bigler, R. D., Y. Bushkin, and N. Chiorazzi. 1988. S152(CD27): a modulating disulfide-linked T cell activation antigen. *J. Immunol. 141:21.*
3. Sugita, K., Y. Torimoto, Y. Nojima, J. F. Daley, S. F. Schlossman, and C. Morimoto. 1991. The 1A4 molecule (CD27) is involved in T cell activation. *J. Immunol. 147:1477.*
4. Camerini, D., G. Walz., W. A. M. Loenen, J. Borst, and B. Seed. 1991. The T cell activation antigen CD27 is a member of the nerve growth factor/tumor necrosis factor receptor gene family. *J. Immunol. 147:3165.*

Downloaded from http://journals.aai.org/jimmunol/article-pdf/161/9/4753/1094561/im2198004753o.pdf by guest on 01 June 2023

5. Loetscher, H., Y. C. Pan, H. W. Lahm, R. Gentz, M. Brockhaus, H. Tabuchi, and W. Lesslauer. 1990. Molecular cloning and expression of the human 55 kD tumor necrosis factor receptor. *Cell 68:351.*
6. Schall, T. J., M. Lewis, K. J. Koller, A. Lee, G. C. Rice, G. H. W. Wong, T. Gatanaga, G. A. Granger, R. Lentz, H. Raab, et al. 1990. Molecular cloning and expression of a receptor for human tumor necrosis factor. *Cell 61:361.*
7. Smith, C. A., T. Davis, D. Anderson, L. Solam, M. P. Beckmann, R. Jerzy, S. K. Dower, D. Cosman, and R. G. Goodwin. 1990. A receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins. *Science 248:1019.*
8. Johnson, D., A. Lanahan, C. R. Buck, A. Sehgal, C. Morgan, E. Mercer, M. Bothwell, and M. Chao. 1986. Expression and structure of the human NGF receptor. *Cell 47:545.*
9. Radeke, M. J., T. P. Misko, C. Hsu, L. A. Herzenberg, and E. M. Shooter. 1987. Gene transfer and molecular cloning of the rat nerve growth factor receptor. *Nature 325:593.*
10. Mallett, S., and A. N. Barclay. 1991. A new superfamily of cell surface proteins related to the nerve growth factor receptor. *Immunol. Today 12:220.*
11. Paulie, S., B. Ehlin-Henriksson, H. Mellstedt, H. Koho, H. Ben-Aissa, and P. Perlmann. 1985. A p50 surface antigen restricted to human urinary bladder carcinomas and B lymphocytes. *Cancer Immunol. Immunother. 20:23.*
12. Clark, E. A., and J. A. Ledbetter. 1986. Activation of human B cells mediated through two distinct cell surface differentiation antigens, Bp35 and Bp50. *Proc. Natl. Acad. Sci. USA 83:4494.*
13. Banchereau, J., and F. Rousset. 1991. Growing human B lymphocytes in the CD40 system. *Nature 353:678.*
14. Stamenkovic, I., E. A. Clark, and B. Seed. 1989. A B-lymphocyte activation molecule related to the nerve growth factor receptor and induced by cytokines in carcinomas. *EMBO J. 8:1403.*
15. Durkop, H., U. Latza, M. Hummel, F. Eitelbach, B. Seed, and H. Stein. 1992. Molecular cloning and expression of a new member of the nerve growth factor receptor family that is characteristic for Hodgkin's disease. *Cell 68:421.*
16. Ellis, T. M., P. E. Simms, D. J. Slivnick, H. M. Jack, and R. I. Fisher. 1993. CD30 is a signal-transducing molecule that defines a subset of human activated CD45RO$^+$ T cells. *J. Immunol. 151:2380.*
17. Itoh, N., S. Yonehara, A. Ishii, M. Yonehara, S. Mizushima, M. Sameshima, A. Hase, Y. Seto, and S. Nagata. 1991. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell 66:233.*
18. Oehm, A., I. Behrmann, W. Falk, M. Pawlita, G. Maier, C. Klas, M. Li-Weber, S. Richards, J. Dhein, B. C. Trauth, et al. 1992. Purification and molecular cloning of the APO-1 cell surface antigen, a member of the tumor necrosis factor/nerve growth factor receptor superfamily: sequence identity with the Fas antigen. *J. Biol. Chem. 267:10709.*
19. Gravestein, L. A., B. Blom, L. A. Nolten, E. de Vries, G. van der Horst, F. Ossendorp, J. Borst, and W. A. M. Loenen. 1993. Cloning and expression of murine CD27: comparison with 4–1BB, another lymphocyte-specific member of the nerve growth factor receptor family. *Eur. J. Immunol. 23:943.*
20. Martorell, J., I. Rojo, R. Vilella, E. Martinez-Caceres, and J. Vives. 1990. CD27 induction on thymocytes. *J. Immunol. 145:1356.*
21. Maurer, D., G. F. Fischer, I. Fae, O. Majdic, K. Stuhlmeier, N. V. Jeney, W. Holter, and W. Knapp. 1992. IgM and IgG but not cytokine secretion is restricted to the CD27$^+$ B lymphocyte subset. *J. Immunol. 148:3700.*
22. Maurer, D., W. Holter, O. Majdic, G. F. Fischer, and W. Knapp. 1990. CD27 expression by a distinct subpopulation of human B lymphocytes. *Eur. J. Immunol. 20:2679.*
23. Sugita, K., M. J. Robertson, J. Ritz, S. F. Schlossman, and C. Morimoto. 1992. Participation of the CD27 antigen in the regulation of IL-2-activated human natural killer cells. *J. Immunol. 149:1199.*
24. Hintzen, R. Q., R. de Jong, S. M. A. Lens, M. Brouwer, P. Baars, and R. A. W. van Lier. 1993. Regulation of CD27 expression on subsets of mature T-lymphocytes. *J. Immunol. 151:2426.*
25. Goodwin, R. G., M. R. Alderson, C. A. Smith, R. J. Armitage, T. VandenBos, R. Jerzy, T. W. Tough, M. A. Schoenborn, T. Davis-Smith, K. Hennen, et al. 1993. Molecular and biological characterization of a ligand for CD27 defines a new family of cytokines with homology to tumor necrosis factor. *Cell 73:447.*
26. Bowman, M. R., M. A. Crimmins, J. Yetz-Aldape, R. Kriz, K. Kelleher, and S. Herrmann. 1994. The cloning of CD70 and its identification as the ligand for CD27. *J. Immunol. 152:1756.*
27. Hintzen, R. Q., S. M. A. Lens, G. Koopman, S. T. Pals, H. Spits, and R. A. W. van Lier. 1994. CD70 represents the human ligand for CD27. *Int. Immunol. 6:477.*
28. Hinzen, R. Q., S. M. A. Lens, K. Lammers, H. Kuiper, M. P. Beckmann, and R. A. W. van Lier. 1995. Engagement of CD27 with its ligand CD70 provides a second signal for T cell activation. *J. Immunol. 154:2612.*
29. Kobata, T., S. Jacquot, S. Kozlowski, K. Agematsu, S. F. Schlossman, and C. Morimoto. 1995. CD27-CD70 interactions regulate B-cell activation by T cells. *Proc. Natl. Acad. Sci. USA 92:11249.*
30. Agematsu, K., T. Kobata, F. C. Yang, T. Nakazawa, K. Fukushima, M. Kitahara, T. Mori, K. Sugita, C. Morimoto, and A. Komiyama. 1995. CD27/CD70 interaction directly drives B cell IgG and IgM synthesis. *Eur. J. Immunol. 25:2825.*
31. Brown, G. R., K. Meek, Y. Nishioka, and D. L. Thiele. 1995 CD27-CD27 ligand/CD70 interactions enhance alloantigen-induced proliferation and cytolytic activity in CD8$^+$ T lymphocytes. *J. Immunol. 154:3686.*
32. Rothe, M., S. C. Wong, W. J. Henzel, and D. V. Goeddel. 1994. A novel family of putative signal transducers associated with the cytoplasmic domain of the 75kDa tumor necrosis factor receptor. *Cell 78:681.*
33. Cheng, G., A. M. Cleary, Z. S. Ye, D. I. Hong, S. Lederman, and D. Baltimore. 1995. Involvement of CRAF1, a relative of TRAF, in CD40 signaling. *Science 267:1494.*
34. Hu, H. M., K. O'Rourke, M. S. Boguski, and V. M. Dixit. 1994. A novel RING finger protein interacts with the cytoplasmic domain of CD40. *J. Biol. Chem. 269:30069.*
35. Sato, T., S. Irie, and J. C. Reed. 1995. A novel member of the TRAF family of putative signal transducing proteins binds to the cytosolic domain of CD40. *FEBS Lett. 358:113.*
36. Regnier, C. H., C. Tomasetto, C. Moog-Lutz, M. P. Chenard, C. Wendling, P. Basset, and M. C. Rio. 1995. Presence of a new conserved domain in CART1, a novel member of the tumor necrosis factor receptor-associated protein family, which is expressed in breast carcinoma. *J. Biol. Chem. 270:25715.*
37. Ishida, T. K., T. Tojo, T. Aoki, N. Kobayashi, T. Ohishi, T. Watanabe, T. Yamamoto, and J. Inoue. 1996. TRAF5, a novel tumor necrosis factor receptor-associated factor family protein, mediates CD40 signaling. *Proc. Natl. Acad. Sci. USA 93:9437.*
38. Nakano, H., H. Oshima, W. Chung, L. Williams-Abbott, C. F. Ware, H. Yagita, and K. Okumura. 1996. TRAF5, an activator of NF-κB and putative signal transducer for the lymphotoxin-beta receptor. *J. Biol. Chem. 271:14661.*
39. Cao, Z., J. Xiong, M. Takeuchi, T. Kurama, and D. V. Goeddel. 1996. TRAF6 is a signal transducer for interleukin-1. *Nature 383:443.*
40. Rothe, M., M. G. Pan, W. J. Henzel, T. M. Ayres, and D. V. Goeddel. 1995. The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. *Cell 83:1243.*
41. Lee, S. Y., G. Kandala, M. L. Liou, H. C. Liou, and Y. Choi. 1996. CD30/TNF receptor-associated factor interaction: NF-κB activation and binding specificity. *Proc. Natl. Acad. Sci. USA 93:9699.*
42. Rothe, M., V. Sarma, V. M. Dixit, and D. V. Goeddel. 1995. TRAF2-mediated activation of NF-κB by TNF receptor 2 and CD40. *Science 269:1424.*
43. Hsu, H., J. Xiong, and D. V. Goeddel. 1995. The TNF receptor 1-associated protein TRADD signals cell death and NF-κB activation. *Cell 81:495.*
44. Hsu, H., H. B. Shu, M. G. Pan, and D. V. Goeddel. 1996. TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell 84:299.*
45. Malinin, N. L., M. P. Boldin, A. V. Kovalenko, and D. Wallach. 1997. MAP3K-related kinase involved in NFκB induction by TNF, CD95 and IL-1. *Nature 385:540.*
46. Natoli, G., A. Costanzo, F. Moretti, M. Fulco, C. Balsano, and M. Levrero. 1997. Tumor necrosis factor (TNF) receptor 1 signaling downstream of TNF receptor-associated factor 2. *J. Biol. Chem. 272:26079.*
47. Woronicz, J. D., X. Gao, Z. Cao, M. Rothe, and D. V. Goeddel. 1997. IκB kinase-β: NF-κB activation and complex formation with IκB kinase-α and NIK. *Science 278:866.*
48. Mercurio, F., H. Zhu, B. W. Murray, A. Shevchenko, B. L. Bennett, J. w. Li, D. B. Young, M. Barbosa, and M. Mann. 1997. IKK-1 and IKK-2: cytokine-activated IκB kinases essential for NFκB activation. *Science 278:860.*
49. Regnier, C. H., H. Y. Song, X. Gao, D. V. Goeddel, Z. Cao, and M. Rothe. 1997. Identification and characterization of an IκB kinase. *Cell 90:373.*
50. Gedrich, R. W., M. C. Gilfillan, C. S. Duckett, J. L. Van Dongen, and C. B. Thompson. 1996. CD30 contains two binding sites with different specificities for members of the tumor necrosis factor receptor-associated factor family of signal transducing proteins. *J. Biol. Chem. 271:12852.*
51. Duckett, C. S., R. W. Gedrich, M. C. Gilfillan, and C. B. Thompson. 1997. Induction of nuclear factor κB by the CD30 receptor is mediated by TRAF1 and TRAF2. *Mol. Cell. Biol. 17:1535.*
52. Seed, B., and A. Aruffo. 1987. Molecular cloning of the CD2 antigen, the T-cell erythrocyte receptor, by a rapid immunoselection procedure. *Proc. Natl. Acad. Sci. USA 84:3365.*
53. Fujita, T., G. P. Nolan, H.-C. Liou, M. L. Scott, and D. Baltimore. 1993. The candidate proto-oncogene *bcl-3* encodes a transcriptional coactivator that activates through NF-κB p50 homodimers. *Genes Dev. 7:1354.*
54. Schreiber, E., P. Matthias, M. M. Muller, and W. Schaffner. 1989. Rapid detection of octamer binding proteins with 'mini-extracts' prepared from a small number of cells. *Nucleic Acids Res. 17:6419.*
55. Inui, S., T. Kaisho, H. Kikutani, I. Stamenkovic, B. Seed, E. A. Clark, and T. Kishimoto. 1990. Identification of the intracytoplasmic region essential for signal transduction through a B cell activation molecule, CD40. *Eur. J. Immunol. 20:1747.*
56. Ishida, T., N. Kobayashi, T. Tojo, S. Ishida, T. Yamamoto, and J. Inoue. 1995. CD40 signaling-mediated induction of Bcl-XL, Cdk4, and Cdk6: implication of their cooperation in selective B cell growth. *J. Immunol. 155:5527.*
57. Boucher, L.-M., L. E. M. Marengere, Y. Lu, S. Thukral, and T. W. Mak. 1997. Binding sites of cytoplasmic effectors TRAF1, 2, 3 on CD30 and other members of the TNF receptor superfamily. *Biochem. Biophys. Res. Commun. 233:592.*
58. Kobata, T., K. Agematsu, J. Kameoka, S. F. Schlossman, and C. Morimoto. 1994. CD27 is a signal-transducing molecule involved in CD45RA$^+$ naive T cell costimulation. *J. Immunol. 153:5422.*

Downloaded from http://journals.aai.org/jimmunol/article-pdf/161/9/4753/1094561/im2198004753o.pdf by guest on 01 June 2023