THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 10, Issue of March 6, pp. 5808–5814, 1998
Printed in U.S.A.

Activation of OX40 Signal Transduction Pathways Leads to Tumor Necrosis Factor Receptor-associated Factor (TRAF) 2- and TRAF5-mediated NF-κB Activation*

(Received for publication, August 4, 1997, and in revised form, December 22, 1997)

Shin Kawamata, Toshiyuki Hori, Akihiro Imura, Akifumi Takaori-Kondo, and Takashi Uchiyama‡

From the Institute for Virus Research, Kyoto University, Kyoto 606, Japan

We investigated the intracellular signaling of OX40, a member of the tumor necrosis factor receptor family. Activation of NF-κB in OX40-transfected HSB-2 cells was detected by electrophoretic mobility shift assay within 30 min after the binding of the ligand gp34. *In vitro* binding experiments showed that tumor necrosis factor receptor-associated factor (TRAF) 1, TRAF2, TRAF3, and TRAF5 but not TRAF4 associated with glutathione *S*-transferase-OX40 fusion protein. The cotransfection experiments using human embryo kidney cell derived HEK 293T cells showed that TRAF2, TRAF3, and TRAF5 associated with OX40 *in vivo*. Studies with OX40 deletion mutants demonstrated that the cytoplasmic portion consisting of amino acid sequence 256–263 (GGSFRTPI) was required for the association with TRAFs and NF-κB activation. The introduction of the dominant negative mutants of TRAF2 and TRAF5 into HSB-2-OX40 cells suppressed NF-κB activation in a dose-dependent manner. In addition, the introduction of TRAF3 together with the dominant negative mutants of TRAF2 or TRAF5 further reduced NF-κB activation. These results indicate that the NF-κB activation resulting from OX40 stimulation is mediated by both TRAF2 and TRAF5, and is likely to be negatively modulated by TRAF3.

Human OX40 is a 50-kDa cell surface glycoprotein expressed primarily on activated $CD4^+$ T cells and some human T cell leukemia virus type I (HTLV-I)[1]-infected T cell lines, but not on resting peripheral T cells, peripheral B cells, or thymocytes. OX40 was originally described as a cell surface antigen on the activated rat T cells (1). Molecular cloning of its cDNA (1–4) revealed that OX40 is a member of the nerve growth factor receptor/tumor necrosis factor receptor (NGF-R/TNF-R) superfamily which is now known to include low affinity nerve growth factor receptor (p75 NGF-R), tumor necrosis factor receptors (p50/55 TNF-R1 and p75/80 TNF-R2), lymphotoxin-β receptor, Fas antigen (CD95/APO-1), CD40, CD30, CD27, and 4-1 BB (5, 6). All the members of this superfamily share a characteristic repeating cysteine-rich motif in the extracellular domain, which is believed to be related to their ability to interact with the TNF-related ligands. The diverse cellular responses such as cell growth, differentiation, and programmed cell death (apoptosis) are triggered by the interaction between the members of the NGF-R/TNF-R superfamily and their ligands.

The ligand for human OX40 was also cloned and identified as previously reported gp34, a cell surface protein expressed on HTLV-I-infected T cell lines and subsequently demonstrated to be induced by transactivator $p40^{tax}$ of HTLV-I (7–9). As expected, the deduced amino acid sequence of gp34 revealed that it is a member of the TNF family. Gp34 has been reported to be expressed on some HTLV-I-infected cell lines such as Hut 102 and MT-2 (10), human umbilical vein endothelial cells (11), and stimulated B lymphoblastoid cell line MSAB (12).

Since its first description, OX40 has been known to transmit costimulatory signals to T cells. Recent studies with human T cells have confirmed this finding and showed that the binding of gp34 to OX40 results in enhanced T cell proliferation and induction of interleukin-2 and -4 production in the presence of anti-CD3 or anti-T cell receptor-αβ antibody (7, 8). We recently reported that the OX40/gp34 system directly mediates the adhesion of activated or HTLV-I-transformed T cells to vascular endothelial cells (11, 13). Furthermore, we examined the role of the OX40/gp34 system in the development of angitis related diseases such as systemic lupus erythematosus and erythema nodosum (14). Although these observations have served to delineate OX40 as a multifunctional cell surface molecule, its physiological as well as pathophysiological significance in viral infection, inflammation (15), or malignant cell infiltration has been poorly defined. In particular, intracellular signaling of OX40 has not been described to date.

Recently, several types of intracellular signal transducer proteins that bind to the members of the TNF-R family and initiate distinct signal transduction have been identified. For example, Fas (CD95) and TNF-R1 are reported to recruit FADD(MORT1)/RIP and TRADD/FADD(MORT1)/RIP, respectively, to initiate signal transduction pathways leading to cell death (16–22). CD30, CD40, TNF-R1, TNF-R2, and lymphotoxin-β receptor recruit several members of a second class of signal transducer family called TRAFs (TNF-R-associated factors). Some members of the TRAF family are responsible for the activation of NF-κB (23–35) or c-Jun N-terminal kinase (36). However, the precise mechanism of TRAF-mediated NF-κB activation (37, 38) or c-Jun N-terminal kinase activation remains unclear.

In the present study, we examined the intracellular events of OX40 signaling using a unique coculture system of OX40-transfected T cells and gp34-transfected adherent cells. We here demonstrate that the OX40 stimulation leads to TRAF-

* This study was supported in part by grants from the Japanese Ministry of Education, Science, Sports and Culture. 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.

‡ To whom correspondence should be addressed: Institute for Virus Research, Kyoto University, 53 Shogoin-kawaracho, Sakyo-ku, Kyoto 606 Japan. Tel.: 81-75-751-4048; Fax: 81-75-751-4049.

[1] The abbreviations used are: HTLV-I, human T cell leukemia virus type I; NGF, nerve growth factor; TNF, tumor necrosis factor; R, receptor; TRAF, tumor necrosis factor receptor-associated factor; RT, reverse transcriptase; PCR, polymerase chain reaction; HA, hemagglutinin; luc, luciferase; CMV, cytomegalovirus; HEK, human embryo kidney; EMSA, electromobility shift assay; PAGE, polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase.

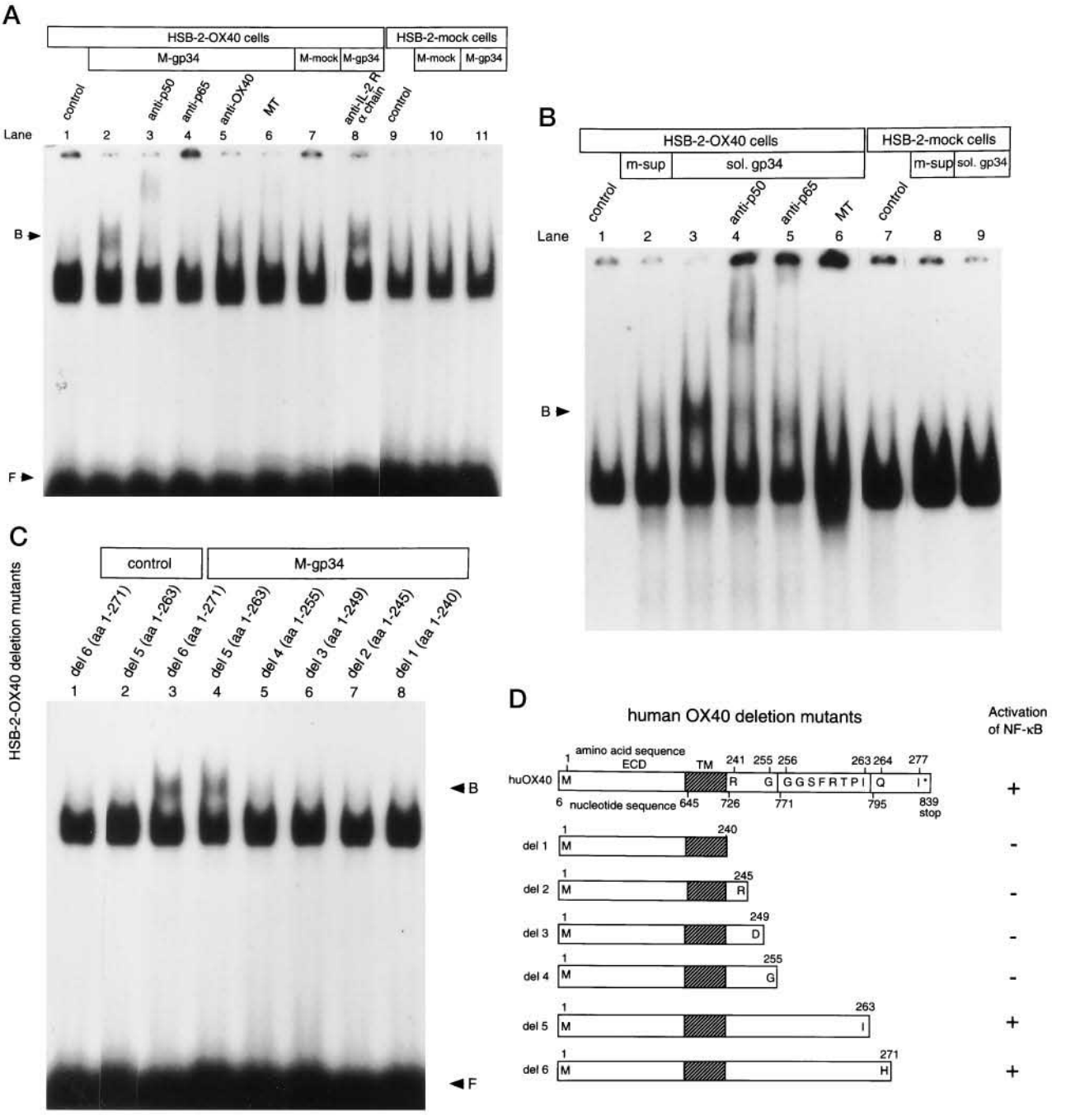

FIG. 1. **NF-κB activation by OX40 stimulation with gp34-transfected MMCE cells (*A*) or soluble gp34 (*B*), and NF-κB activating capability of various OX40 deletion mutants (*C* and *D*).** Eight μg of the nuclear extracts of HSB-2-OX40 cells or HSB-2-mock cells were prepared after they were cocultured on the monolayer of MMCE-gp34 cells (*M-gp34*), MMCE-mock cells (*M-mock*), or cultured with soluble gp34-sup (*sol. gp34*), mock-sup (*m-sup*) for 30 min. The samples were analyzed by EMSA with κB consensus oligonucleotide except for *lane 6* of *A* and *B*, where κB mutant oligonucleotide (*MT*) was used. *A*, NF-κB activation by stimulation with MMCE-gp34. HSB-2-OX40 cells (*lanes 1–8*) or HSB-2-mock cells (*lanes 9–11*) were cultured with medium alone (*lanes 1* and *9*), MMCE-gp34 (*lanes 2–6*, *8*, and *11*) or MMCE-mock (*lanes 7* and *10*). In *lanes 5* and *8*, cells were preincubated with anti-OX40 antibody (*lane 5*) or with anti-interleukin-2Rα chain antibody (*lane 8*) for 30 min prior to coculture. The nuclear extracts from HSB-2-OX40 cells cocultured with MMCE-gp34 cells were incubated with anti-NF-κB p50 antibody (*lane 3*) or with anti-NF-κB p65 antibody (*lane 4*) for supershift assay. The positions *B* and *F* indicate the inducible NF-κB protein complex and the free fraction of ^{32}P-labeled oligonucleotide, respectively. The band observed between *B* and *F* is a nonspecific nuclear protein of HSB-2-OX40 cells and HSB-2-mock cells. *B*, NF-κB activation by stimulation with soluble gp34. HSB-2-OX40 cells (*lanes 1–6*) or HSB-2 mock cells (*lanes 7–9*) were cultured with medium alone (*lanes 1* and *7*), mock-sup (*lanes 2* and *8*), or soluble gp34 (*lanes 3–6* and *9*). The nuclear extracts from HSB-2-OX40 cells cultured with soluble gp34 for 30 min were incubated with anti-NF-κB p50 antibody (*lane 4*) or with anti-NF-κB p65 antibody (*lane 5*) for supershift assay. The position *B* indicates the inducible NF-κB protein. *C* and *D*, NF-κB activating capability of various OX40 deletion mutants. Various HSB-2-OX40 deletion mutants shown in *D* were cultured with medium alone (*lane 1* and *2*, *control*) or cocultured with MMCE-gp34 cells (*lanes 3–8*) for 30 min and then NF-κB activation was examined by EMSA as described in *A* and *B*. The positions *B* and *F* indicate the inducible NF-κB protein complex and free fraction of ^{32}P-labeled oligonucleotide, respectively.

mediated NF-κB activation. Based on the experimental results, a possible physiological and pathophysiological significance of the OX40 signaling in activated T cells is discussed.

MATERIALS AND METHODS

Preparation of Plasmid Constructs—Based on the published cDNA sequence of human gp34, cDNA of the entire coding region of gp34 was

obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) method. The PCR products were ligated into an expression vector pMKIT Neo (a gift of Dr. K. Maruyama, Tokyo Medical and Dental University) to construct pMKIT Neo-gp34. The preparation of an expression vector, pMKIT Neo-OX40, was described previously (11). The constructs for OX40 cytoplasmic deletion mutants were generated by PCR method using TAG (stop codon)-tagged oligonucleotides as the primer and pMKIT Neo-OX40 as the template. The PCR products were ligated into pMKIT Neo to construct pMKIT Neo-OX40-del 1, -del 2, -del 3, -del 4, -del 5, and -del 6. The partial DNA sequences were determined for all the OX40 deletion mutants to confirm the constructs.

Based on the published cDNA sequences of murine TRAF1, TRAF2, TRAF5, and human TRAF3 and TRAF4, the cDNAs of TRAFs were obtained by RT-PCR. The oligonucleotides covering the entire coding regions of TRAFs and cDNAs from murine cell line DA-1 (for TRAF1, TRAF2, and TRAF5) or phytohemagglutinin-stimulated human peripheral blood mononuclear cells (for TRAF3 and TRAF4) were used as the primers and the templates, respectively. The PCR products were integrated into the expression vector pcDNA3 (Invitrogen, San Diego, CA) or HA-tagged expression vector pCMV4-3 HA^+ (pCMV-HA, a gift of Dr. W. C. Greene, Gladstone Institute of Virology and Immunology, University of California, San Francisco) to construct pcDNA3-TRAFs or pCMV-HA-TRAFs. The cDNAs of truncated TRAF2 (TRAF2 DN) and truncated TRAF5 (TRAF5 DN) were generated by PCR with the primers covering the amino acid sequence (amino acids 256–501) of TRAF2 (21) and (amino acids 233–558) of TRAF5 (35), respectively, and then integrated into pcDNA3.

Preparation of Soluble gp34—A construct for soluble gp34 was designed by fusing the extracellular portion of gp34 (nucleotide sequence, 187–585) to the signal sequence of OX40 (nucleotide sequence, 6–116) at the *Sma*I site. The fused fragments were ligated into the expression vector pME18S (11) (a gift of Dr. K. Maruyama). Four μg of pME18S-soluble gp34 were transfected into COS-7 cells (1×10^7 cells) by the DEAE-dextran method (39). The transfected cells were cultured with Dulbecco's modified Eagle's medium (Life Technologies, Inc.) with 10% fetal calf serum (Bio Whittaker, Verviers, Belgium) for 24 h and with 1% fetal calf serum for another 48 h. The culture supernatants were collected and concentrated 10-fold with Centriprep 10 (Amicon Inc., Beverly, MA) prior to the assay. The supernatant of COS-7 cells transfected with pME18S-soluble gp34 (soluble gp34-sup) was added to the cell culture at 25% v/v for each assay. The supernatant of COS-7 cells transfected with empty vector (mock-sup) was prepared by the same method as soluble gp34-sup and concentrated 10-fold prior to the assay.

Cells and Culture Conditions—Human T cell line HSB-2 and murine epithelial cell line MMCE were cultured in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum, 60 mM tobramycin, and 2 mM L-glutamine. Human embryo kidney cell-derived cell line HEK 293T and COS-7 were cultured with Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

The stable transfectants of OX40 (HSB-2-OX40 cells), or OX40 deletion mutants (HSB-2-OX40-del 1, -del 2, -del 3, -del 4, -del 5, or -del 6 cells) were prepared by introducing pMKIT Neo-OX40, or pMKIT Neo-OX40-del 1, -del 2, -del 3, -del 4, -del 5, or -del 6 into HSB-2 cells by the electroporation method. The stable transfectants of gp34 (MMCE-gp34 cells) were prepared by introducing pMKIT Neo-gp34 into MMCE cells by the electroporation method. The transfected cells were dispersed in 96-well flat-bottomed plates for clonal selection and cultured in RPMI 1640 medium containing 1 mg/ml G418 (Sigma) for 3 weeks. The expression of OX40 or gp34 on the transfected cells was examined with a FACScan (Becton Dickinson, San Jose, CA) prior to the assay. The empty vector was transfected into HSB-2 cells or MMCE cells to prepare HSB-2-mock cells or MMCE-mock cells. The empty vector transfected cells (mock cells) were cultured in 10-cm dishes with RPMI 1640 medium containing 1 mg/ml G418. The intracellular events in HSB-2-OX40 cells were examined after they were cultured on the monolayer of adhesive MMCE-gp34 cells. The contamination of MMCE-gp34 cells in the harvested cells was evaluated by RT-PCR (94 °C 1 min, 60 °C 1 min, 72 °C 2 min for 25 cycles) using murine specific β-actin primers (forward 5′-CGCTGCGCTGGTCGTCGACA-3′, reverse 5′-CAGCACAGCCTGGATGGCTA-3′) and cDNA from the harvested cells. 0.5% contamination of MMCE-gp34 cells in HSB-2-OX40 cells was detected under the PCR conditions stated above.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts from HSB-2-OX40 cells or HSB-2-mock cells (2×10^6 cells) cocultured with either MMCE-gp34 cells or MMCE-mock cells, or cultured with either soluble gp34-sup or mock-sup for the indicated periods were prepared as described previously (40). The nuclear extracts from HSB-2-OX40 cells preincubated (at 37 °C for 30 min) with either anti-OX40 monoclonal antibody (50 μg/ml) (11) or anti-interleukin-2 receptor α chain antibody (anti-Tac, control antibody, 50 μg/ml) prior to coculture with MMCE-gp34 cells were also prepared. Eight μg of nuclear extracts were mixed with ^{32}P-labeled κB oligonucleotide containing a binding site for NF-κB/c-Rel homodimeric and heterodimeric complexes (5′-AGTTGAGGGGACTTTCCCAGGC-3′) (Santa Cruz Biotechnology, Santa Cruz, CA) or ^{32}P-labeled mutant κB oligonucleotide (5′-AGTTGAGGCGACTTTCCCAGGC-3′) (Santa Cruz). The binding assay was performed as described previously with a slight modification (41). The composition of the induced NF-κB complex was examined by super shift assay with anti-NF-κB p50 subunit antibody or anti-NF-κB p65 subunit antibody (Upstate Biotechnology Inc., Lake Placid, NY). Eight μg of nuclear extracts in 10 μl of nuclear extract buffer (40) was incubated with 100 ng of anti-NF-κB antibodies at room temperature for 40 min prior to the binding assay.

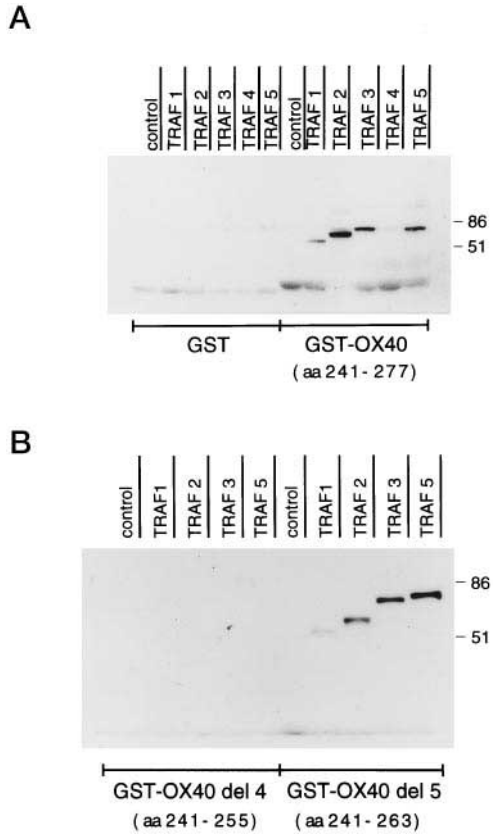

FIG. 2. **Association of TRAFs with OX40 *in vitro*.** The cell lysates prepared from HEK 293T cells transfected with pCMV-HA-TRAF1, -TRAF2, -TRAF3, -TRAF4, -TRAF5, or pCMV-HA were mixed with either GST-OX40- (the entire cytoplasmic portion, amino acids 241–277), GST-OX40-del 4 (amino acids 241–255), GST-OX40-del 5 (amino acids 241–263), or GST-Sepharose beads. Protein bound to GST-OX40 or GST was analyzed by SDS-PAGE with 7.5% gel and subjected to immunoblotting with anti-HA antibody (*A*). Protein bound to GST-OX40-del 4 or GST-OX40-del 5 was analyzed by SDS-PAGE with 10.5% gel and subjected to immunoblotting with biotin-conjugated anti-HA antibody (*B*).

Coimmunoprecipitation and in Vivo Binding Assay—Two μg of pCMV-HA-TRAF1, -TRAF2, -TRAF3, -TRAF4, -TRAF5, or pCMV-HA were cotransfected with 2 μg of pME18S-OX40 or pME18S into HEK 293T (2×10^5 cells) by the calcium phosphate precipitation method (26). After 48 h of incubation, the cells were lysed in the Triton X lysing buffer (0.5% Triton X-100, 25 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 10% glycerol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 2 μg/ml pepstatin A). The cell lysates were immunoprecipitated with protein A-Sepharose 4FF (Pharmacia Biotech Inc., Uppsala, Sweden) and anti-OX40 monoclonal antibody (11). The immunoprecipitates were analyzed by SDS-PAGE with 7.5% gel (ATTO, Tokyo, Japan) and subjected to immunoblotting (11) with anti-HA monoclonal antibody, 12CA5 (Boehringer Mannheim). HA-tagged TRAFs were visualized by ECL detection system (Amersham Life Science, Arlington Heights, IL). The same membrane was

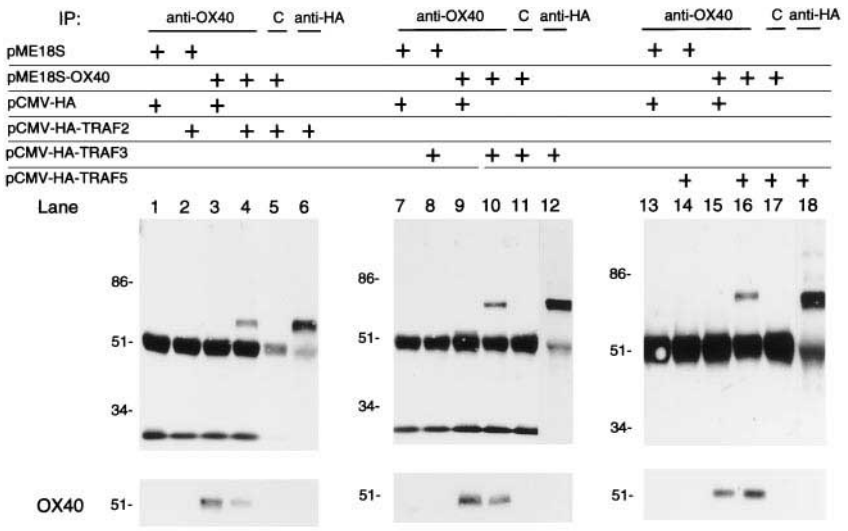

FIG. 3. **Association of TRAFs with OX40 *in vivo*.** An expression vector encoding OX40, pME18S-OX40, or pME18S was cotransfected into HEK 293T cells with pCMV-HA-TRAF2, -TRAF3, -TRAF5, or pCMV-HA. After 48 h of incubation, cell lysates were prepared and subjected to immunoprecipitation (*IP*) with anti-OX40 monoclonal antibody, IgG subclass-matched control antibody (*C*), or anti-HA polyclonal antibodies. The samples were analyzed by SDS-PAGE with 7.5% gel, followed by immunoblotting with anti-HA monoclonal antibody. The same membrane was reused for the detection of OX40 by immunoblotting. The bands near 51 kDa and below 34 kDa are mouse IgG heavy chain and light chain, respectively. The association of OX40 with TRAF2, TRAF3, and TRAF5 is shown in *lanes 1–6*, *lanes 7–12*, and *lanes 13–18*, respectively.

reused for the detection of OX40 by immunoblotting.

Glutathione S-Transferase (GST) Fusion Protein Expression and in Vitro Binding Assay—The cDNA of cytoplasmic portion of OX40 (corresponding to amino acids 241–277), OX40-del 3 (amino acids 241–249), OX40-del 4 (amino acids 241–255), OX40-del 5 (amino acids 241–263), or OX40-del 6 (amino acids 241–271) was inserted into the GST-fusion vector pGEX-5X-1 (Pharmacia). Glutathione-Sepharose 4B beads (Pharmacia) conjugated with GST-OX40, GST-OX40-del 3, GST-OX40-del 4, GST-OX40-del 5, GST-OX40-del 6, or GST were prepared as described previously (42). Four μg of pCMV-HA-TRAF1, -TRAF2, -TRAF3, -TRAF4, -TRAF5, or pCMV-HA was transfected into HEK 293T cells (2×10^5 cells) by the calcium phosphate precipitation method. After 48 h of incubation, the cell lysates were prepared by the same method as stated for the *in vivo* binding assay and mixed with either GST-OX40-, GST-OX40-del 3-, GST-OX40-del 4-, GST-OX40-del 5-, GST-OX40-del 6-, or GST-Sepharose 4B beads by rotating at 4 °C for 6 h. Protein bound to GST or GST-OX40 fusion protein was analyzed by SDS-PAGE with 7.5% gel (ATTO) and subjected to immunoblotting with anti-HA antibody 12CA5. Protein bound to GST-OX40-del 3, GST-OX40-del 4, GST-OX40-del 5, or GST-OX40-del 6 was analyzed by SDS-PAGE with 10.5% gel (ATTO) and subjected to immunoblotting with biotin-conjugated anti-HA-monoclonal antibody (Boehringer Mannheim). HA-tagged TRAFs were visualized by ECL detection system (Amersham).

Luciferase Assay—In the studies of NF-κB activation by overexpression of TRAFs, 1.5 μg of pcDNA3-TRAF1, -TRAF2, -TRAF3, -TRAF4, -TRAF5, -TRAF2 DN, or -TRAF5 DN were transfected into HSB-2-OX40 cells (1×10^6 cells) together with 500 ng of κB site integrated luciferase reporter gene κB-luc (43) (a gift of Dr. W. Greene) and 250 ng of pCSK-LacZ (27) by the DEAE-dextran method (44). The total amount of pcDNA3 constructs was adjusted to 3 μg by adding an empty vector. After 28 h of incubation the cells were lysed in 250 μl of reporter lysis buffer (Toyo Ink Co., Tokyo Japan). Twenty μl of cell extract from each sample were fractionated to measure the luciferase activity in accordance with the manufacturer's protocol (Toyo Ink) using a luminometer (Bio-Orbit Oy, Turku, Finland). Forty μl of cell extract were fractionated to measure β-galactosidase activity as an internal control (27). The luminescence values were normalized by the individual β-galactosidase activity. In the experiments of TRAF2- and TRAF5-mediated NF-κB activation, pcDNA3-TRAF2 DN, -TRAF5 DN, -TRAF3, or pcDNA3 was cotransfected with 500 ng of κB-luc and 250 ng of pCSK-LacZ into HSB-2-OX40 cells or HSB-2 mock cells (1×10^6 cells) by the DEAE-dextran method. The total amount of pcDNA3 constructs was adjusted to 3 μg by adding an empty vector. After 24 h of incubation, the transfected cells were cocultured with either MMCE-gp34 cells or MMCE-mock cells for 24 h and then harvested to measure luciferase activity and β-galactosidase activity as described above.

RESULTS

NF-κB Is Activated by OX40 Stimulation—Activation of NF-κB in HSB-2-OX40 cells was detected by EMSA when the cells were cocultured with MMCE-gp34 cells but not with MMCE-mock cells (Fig. 1*A*). The activation was detected from 30 min up to 6 h (the end of culture period, data not shown) after ligand stimulation and blocked clearly by preincubation of HSB-2-OX40 cells with anti-OX40 antibody. The supershift of the band with anti-NF-κB p50 subunit antibody or anti-NF-κB p65 subunit antibody indicated the involvement of NF-κB, consisting of p50 and p65 subunits in OX40-mediated activation. Similar results were obtained when the HSB-2-OX40 cells were incubated with soluble gp34-sup, but not with mock-sup (Fig. 1*B*). Furthermore, the studies with HSB-2-OX40 deletion mutants demonstrated that NF-κB activation was detected in HSB-2-OX40-del 5 (amino acids 1–263) cells and -OX40-del 6 (amino acids 1–271) cells, but not HSB-2-OX40-del 4 (amino acids 1–255) cells after ligand stimulation. These results indicated that the cytoplasmic portion of OX40 consisting of the amino acid sequence 256–263 (GGSFRTPI) was required for the activation of NF-κB (Fig. 1, *C* and *D*).

TRAF 1, TRAF2, TRAF3, and TRAF5 but Not TRAF 4 Associate with OX40 in Vitro—It has been reported that TRAFs associate with the receptors of several members of the TNF-R family and initiate the signal transduction upon the ligand stimulation. We examined the association of TRAFs with OX40 using HA-tagged-TRAFs and GST-OX40 fusion protein. As shown in Fig. 2*A*, HA-TRAF1, -TRAF2, -TRAF3, and -TRAF5 but not HA-TRAF4 associated with GST-OX40. HA-TRAF1, -TRAF2, -TRAF3, -TRAF4, and -TRAF5 were successfully expressed in HEK293 T cells and immunoprecipitated with anti-HA antibody (data not shown). Furthermore, the studies with GST-OX40 deletion mutants demonstrated that TRAF1, TRAF2, TRAF3, and TRAF5 associated with GST-OX40-del 5 (amino acids 241–263) and GST-OX40-del 6 (amino acids 241–271) (data not shown), but not with GST-OX40-del 3 (amino acids 241–249) (data not shown) or GST-OX40-del 4 (amino acids 241–255) *in vitro* (Fig. 2*B*). In other words, the cytoplasmic portion of OX40 consisting of the amino acid sequence 256–263 (GGSFRTPI) was required for the association with TRAF1, TRAF2, TRAF3, and TRAF5 *in vitro*.

TRAF2, TRAF3, and TRAF5 Associate with OX40 in Vivo—We next examined the association of TRAFs with OX40 *in vivo*. HA-TRAF2, HA-TRAF3, and HA-TRAF5 were coimmunoprecipitated with anti-OX40 antibody when coexpressed with OX40 in HEK 293T cells, indicating that these TRAFs can associate with OX40 *in vivo* (Fig. 3). HA-TRAF1 and HA-TRAF4 were successfully expressed in HEK 293T cells and immunoprecipitated with anti-HA antibody. However, the association of HA-TRAF1 or HA-TRAF4 with OX40 *in vivo* was not detected under this condition (data not shown).

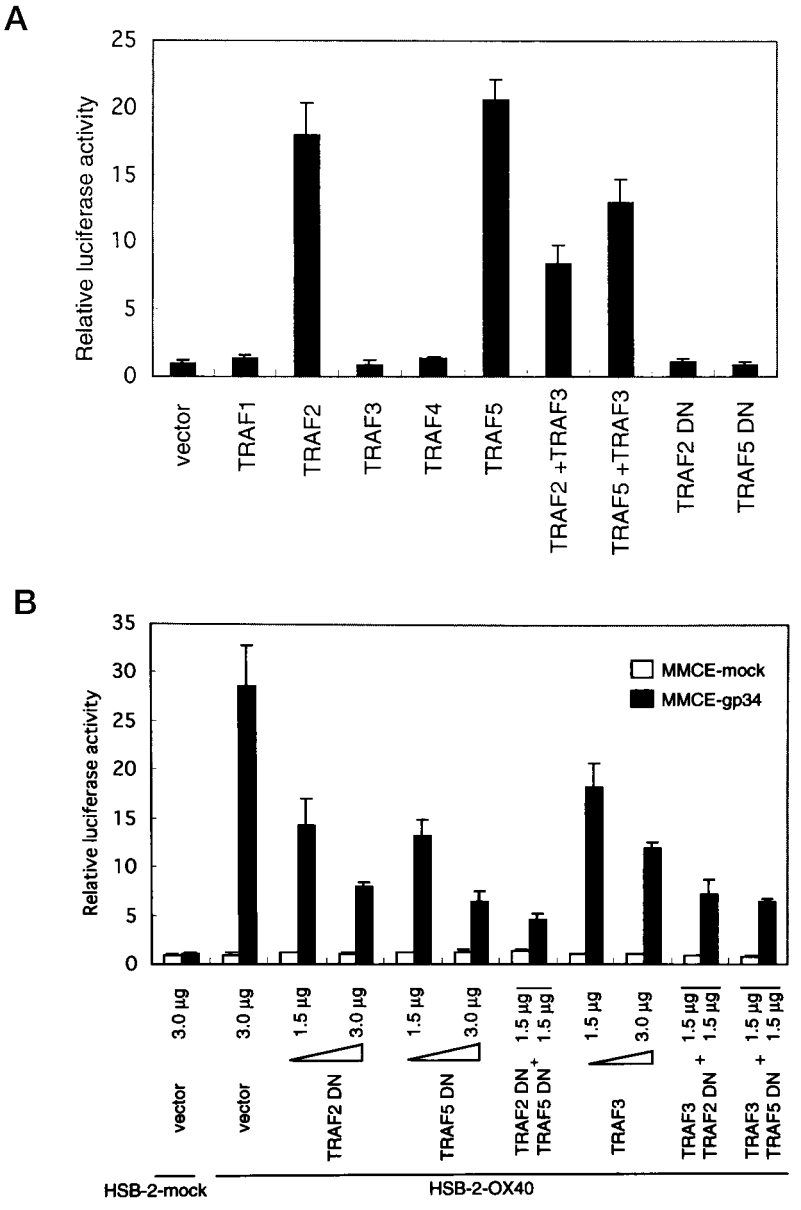

FIG. 4. *A*, NF-κB activation by overexpression of TRAFs in HSB-2-OX40 cells. One and a half μg of pcDNA3-TRAF1, -TRAF2, -TRAF3, -TRAF4, -TRAF5, -TRAF2 DN (amino acids 256–501), or -TRAF5 DN (amino acids 233–558) was transfected together with 500 ng of the luciferase reporter plasmid κB-luc and 250 ng of pCSK-LacZ into HSB-2-OX40 cells. The total amount of pcDNA3 constructs was adjusted to 3 μg by adding an empty vector. After 28 h of incubation, cell lysates were prepared and subjected to luciferase assay. All values representing luciferase activities were normalized by individual β-galactosidase activities and are shown as the means of the triplicate samples with ±S.D. *B*, suppression of NF-κB activation by TRAF2 DN, TRAF5 DN, and TRAF3. pcDNA3-TRAF2 DN, -TRAF5 DN, -TRAF3, or pcDNA3 was transfected into HSB-2-OX40 cells or HSB-2-mock cells together with κB-luc and pCSK-LacZ. After 24 h of incubation, transfected cells were cocultured with either MMCE-gp34 cells or MMCE-mock cells for another 24 h, and then luciferase activity was determined as described in *A* and under "Materials and Methods."

TRAF2 and TRAF5 Mediate NF-κB Activation in OX40 Signaling, while TRAF3 Negatively Modulates NF-κB Activation—To evaluate the ability of various TRAFs to mediate NF-κB activation in HSB-2-OX40 cells, pcDNA3-TRAFs were transfected with the luciferase reporter plasmid κB-luc into HSB-2-OX40 cells. The luciferase assay of the transfected cell lysates demonstrated that TRAF2 and TRAF5 but not TRAF1, TRAF3, or TRAF4 were able to mediate NF-κB activation when overexpressed in HSB-2-OX40 cells (Fig. 4*A*). Since TRAF2, TRAF3, and TRAF5 were found to associate with OX40 *in vivo*, we examined the effects of TRAF3 on NF-κB activation mediated by TRAF2 or TRAF5 in HSB-2-OX40 cells. As shown in Fig. 4*A*, the introduction of TRAF3 reduced the levels of NF-κB activation by overexpressed TRAF2 or TRAF5. Based on this experiment, we further examined the roles of TRAF2, TRAF5, and TRAF3 in NF-κB activation resulting from OX40 stimulation by introducing the dominant negative forms of TRAF2 (TRAF2 DN), TRAF5 (TRAF5 DN), or wild type TRAF3 into HSB-2-OX40 cells. The transfected HSB-2-OX40 cells were stimulated by MMCE-gp34 cells. As shown in Fig. 4*B*, the introduction of TRAF2 DN or TRAF5 DN suppressed the luciferase activity in a dose dependent manner. Furthermore, the introduction of both TRAF2 DN and TRAF5 DN suppressed the luciferase activity markedly to the lowest level. The introduction of TRAF3 together with TRAF2 DN or TRAF5 DN reduced NF-κB activation further, which suggests that TRAF3 modulates NF-κB activation negatively in OX40 signaling.

DISCUSSION

In the present study, we demonstrated that TRAF2- and TRAF5-mediated NF-κB activation was induced by OX40 stimulation. We have had difficulties in the study of human OX40 signaling, since neither of our two anti-OX40 monoclonal antibodies (11) could trigger OX40 signaling even when cross-linked with the second antibody. We, therefore, employed a unique coculture system of OX40-transfected HSB-2 cells and human gp34-transfected MMCE cells. The separation of HSB-2-OX40 cells from MMCE-gp34 cells was easy and the contamination of MMCE-gp34 cells in the harvested cells was estimated to be less than 0.5% by RT-PCR method using murine specific primers. We also employed the culture supernatants of COS-7 cells transfected with the soluble gp34-construct in most of the assays to confirm the data obtained from the coculture system. Although the signals triggered by soluble gp34 were

somewhat weaker than those by membrane-bound gp34, the experiments with soluble gp34 gave essentially the same results.

The studies with OX40 deletion mutants demonstrated that the cytoplasmic portion of OX40 consisting of the amino acid sequence 256–263 (GGSFRTPI) was required for association with TRAFs and NF-κB activation. It is notable that the potential phosphorylation site for protein kinase C is included in this portion of 8 amino acid residues (4). In addition, our preliminary studies showed the induction of c-jun mRNA by OX40 stimulation, for which the same intracytoplasmic portion GGSFRTPI was required (data not shown). Taken together, we consider that this portion of 8 amino acid residues constitutes a part of the crucial domain that initiates multiple signal transductions upon OX40 stimulation.

Previous studies demonstrated that several members of TRAFs associate with the members of the TNF-R family and initiate signal transduction. For example, TNF-R2 is associated with TRAF1, TRAF2, or TRAF3 (23–26). TNF-R1 is associated with TRAF2 through TRADD (21). CD40 is associated with TRAF2 (26, 27) or TRAF3 (23, 28–30). CD30 is associated with TRAF1, TRAF2, TRAF3, or TRAF5 (31–34). Lymphotoxin-β receptor is associated with TRAF3 (23) or TRAF5 (35). In some of these reports, two independent motifs in the receptors, EEEGKE and PXQE, have been described as "TRAFs-binding motifs" (31, 32, 45). While the EEEGKE motif that interacts with TRAF1 and TRAF2, but not with TRAF3, is not found in OX40, the PXQE motif that interacts with TRAF2 and TARF3 is conserved among CD30 (amino acids 561–564), CD40 (amino acids 250–253), and OX40 (amino acids 262–265). However, the OX40 deletion mutant HSB-2-OX40 del 5 (amino acids 1–263) cells were able to trigger intracellular signaling leading to NF-κB activation upon ligand stimulation and GST-OX40 del 5 (amino acids 241–263) was able to associate with TRAFs *in vitro*. We, therefore, need further evaluation to determine whether all four amino acid residues (PXQE) are indispensable for the association with TRAFs and NF-κB activation. Several reports indicate that TRAFs are recruited to the receptor by forming the complex with the receptor-associated molecules such as TRADD (21) and/or TRAF-associated molecules such as c-IAP (24) and TRIP (46). It is, therefore, possible that the binding site and the binding affinity between TRAFs and the receptors are affected by such interacting molecules.

In most of the members of the TNF-R family, TRAF2 and/or TRAF5 are responsible for the activation of NF-κB, while the function of TRAF1 (31), TRAF3, or TRAF4 (47) has not been clearly understood. We demonstrated that both TRAF2 and TRAF5 mediated NF-κB activation in OX40 signaling, whereas TRAF3 exerted suppressive effects on NF-κB activation as previously reported in CD30 signaling (31). Further studies will be needed to elucidate the precise role of TRAF3 in OX40 signaling.

TRAF-mediated NF-κB activation, indeed, has furnished a clue to the understanding of the signaling in the several members of the TNF-R family. However, studies of the downstream events after NF-κB activation as well as other signaling pathways would be one of the key issues to be addressed to give a satisfactory explanation of the diverse cellular responses triggered by the stimulation of the members of the TNF-R family. Recently several groups reported that the activation of NF-κB blocked apoptosis (48–50), which may help to understand the role of the OX40/gp34 system *in vivo*. The conspicuous feature of the OX40/gp34 system among the TNF-R/TNF family is its ability to mediate adhesion between activated or HTLV-I-transformed T cells and endothelial cells. It is, therefore, possible that the OX40 signaling in T cells is triggered by the interaction with endothelial cells of the tissues where activated T cells expressing OX40 are infiltrating. OX40-mediated NF-κB activation in T cells may serve to protect them from apoptosis, which results in the amplification and prolongation of the immune responses, or in the case of adult T cell leukemia, prolonged survival of leukemic cells in the infiltrated tissues.

Acknowledgments—We are grateful to Dr. K. Maruyama for providing pMKIT Neo and pME18S expression vectors, and Dr. W. C. Greene for the luciferase reporter plasmid construct κB-luc and HA-tagged vector pCMV-HA. We also thank K. Fukunaga for excellent technical assistance and useful discussions.

REFERENCES

1. Paterson, D. J., Jefferies, W. A., Green, J. R., Brandon, M. R., Corthesy, P., Puklavic, M., and Williams, A. F. (1987) *Mol. Immunol.* **24,** 1281–1290
2. Mallett, S., Fossum, S., and Barclay, A. N. (1990) *EMBO J.* **9,** 1063–1068
3. Calderhead, D. M., Buhlmann, J. E., van den Eertwegh, A. J., Claassen, E., Noelle, R. J., and Fell, H. P. (1993) *J. Immunol.* **151,** 5261–5271
4. Latza, U., Dürkop, H., Schnittger, S., Ringeling, J., Eitelbach, F., Hummel, M., Fonatsch, C., and Stein, H. (1994) *Eur. J. Immunol.* **24,** 677–683
5. Smith, C. A., Farrah, T., and Goodwin, R. G. (1994) *Cell* **76,** 959–962
6. Baker, S. T., and Reddy, E. P. (1996) *Oncogene* **12,** 1–9
7. Baum, P. R., Gayle III, R. B., Ramsdell, F., Srinivasan, S., Sorensen, R. A., Watson, M. L., Seldin, M. F., Baker, E., Sutherland, G. R., Clifford, K. N., Alderson, M. R., Goodwin, R. G., and Fanslow, W. C. (1994) *EMBO J.* **13,** 3992–4001
8. Godfrey, W. R., Fagnoni, F. F., Harara, M. A., Buck, D., and Engleman, E. G. (1994) *J. Exp. Med.* **180,** 757–762
9. Miura, S., Ohtani, K., Numata, N., Niki, M., Ohbo, K., Ina, Y., Gojobori, T., Tanaka, Y., Tozawa, H., Nakamura, M., and Sugamura, K. (1991) *Mol. Cell. Biol.* **11,** 1313–1325
10. Tanaka, Y., T. Inoi, T., Tozawa, H., Yamamoto, N., and Hinuma, Y. (1985) *Int. J. Cancer* **36,** 549–551
11. Imura, A., Hori, T., Imada, K., Ishikawa, T., Tanaka, Y., Maeda, M., Imamura, S., and Uchiyama, T. (1996) *J. Exp. Med.* **183,** 2185–2195
12. Stüber, E., Neurath, M., Calderhead, D., Fell, H. P., and Strober, W. (1995) *Immunity* **2,** 507–521
13. Imura, A., Hori, T., Imada, K., Kawamata, S., Tanaka, Y., Imamura, S., and Uchiyama, T. (1997) *Blood* **89,** 2951–2958
14. Matsumura, Y., Imura, A., Hori, T., Uchiyama, T., and Imamura, S. (1997) *Arch. Dermatol. Res.*, **289,** 653–656
15. Weinberg, A. D., Bourdette, D. N., Sullivan, T. J., Lemon, M., Wallin, J. J., Maziarz, R., Davey, M., Palida, F., Godfrey, W., Engleman, E., Fulton, R. J., Offner, H., and Vandenbark, A. A. (1996) *Nat. Med.* **2,** 183–189
16. Stanger, B. Z., Leder, P., Lee, T.-H., Kim, E., and Seed, B. (1995) *Cell* **81,** 513–523
17. Boldin, M. P., Varfolomeev, E. F., Pancer, Z., Mett, I. L., Camonis, J. H., and Wallach, D. (1995) *J. Biol. Chem.* **270,** 7795–7798
18. Chinnaiyan, A. M., O'Rourke, K., Tewari, M., and Dixit, V. M. (1995) *Cell* **81,** 505–512
19. Hsu, H., Xiong, J., and Goeddel, D. V. (1995) *Cell* **81,** 495–504
20. Hsu, H., Huang, J., Shu, H.-B., Baichwal, V., and Goeddel, D. V. (1996) *Immunity* **4,** 387–396
21. Hsu, H., Shu, H.-B., Pan, M.-G., and Goeddel, D. V. (1996) *Cell* **84,** 299–308
22. Muzio, M., Chinnaiyan, A. M., Kischkel, F. C., O'Rourke, K., Shevchenko, A., J, Ni., Scaffidi, C., Brentz, J. D., Zhang, M., Gentz, R., Matthias, M., Krammer, P. H., Peter, M. E., and Dixit, V. M. (1996) *Cell* **85,** 817–827
23. Mosialos, G., Birkenbach, M., Yalamanchili, R., VanArsdale, T., Ware, C., and Kief, E. (1995) *Cell* **80,** 389–399
24. Rothe, M., Pan, M.-G., Henzel, W. J., Ayres, T. M., and Goeddel, D. V. (1995) *Cell* **83,** 1243–1252
25. Rothe, M., Wong, S. C., Henzel, W. J., and Goeddel, D. V. (1994) *Cell* **78,** 681–692
26. Rothe, M., Sarma, V., Dixit, V. M., and Goeddel, D. V. (1995) *Science* **269,** 1424–1427
27. Cheng, G., and Baltimore, D. (1996) *Genes Dev.* **10,** 963–973
28. Sato, T., Irie, S., and Reed, J. C. (1995) *FEBS Lett.* **358,** 113–118
29. Cheng, G., Cleary, A. M., Ye, Z.-S., Hong, D. I., Lederman, S., and Baltimore, D. (1995) *Science* **267,** 1494–1498
30. Hu, H. M., O'Rourke, K., Boguski, M. S., and Dixit, V. M. (1994) *J. Biol. Chem.* **269,** 30069–30072
31. Duckett, C. S., Gedrich, R. W., Gilfillan, M. C., and Thompson, C. B. (1997) *Mol. Cell. Biol.* **17,** 1535–1542
32. Gedrich, R. W., Gilfillan, M. C., Duckett, C. S., Van Dongen, J. L., and Thompson, C. B. (1996) *J. Biol. Chem.* **271,** 12852–12858
33. Lee, S. Y., Park, C. G. and Choi, Y. (1996) *J. Exp. Med.* **183,** 669–674
34. Aizawa, S., Nakano, H., Ishida, T., Horie, R., Nagai, M. Ito, K., Yagita, H., Okumura, K., Inoue, J., and Watanabe, T. (1997) *J. Biol. Chem.* **272,** 2042–2045
35. Nakano, H., Oshima, H., Chung, W., Williams-Abbott, L., Ware, C. F., Yagita, H., and Okumura, K. (1996) *J. Biol. Chem.* **271,** 14661–14664
36. Liu, Z.-G., Hsu, H., Goeddel, D. V., and Karin, M. (1996) *Cell* **87,** 565–576
37. Malinin, N. L., Boldin, M. P., Kovalenko, A. V., and Wallach, D. (1997) *Nature* **385,** 540–544
38. Regnier, C. H., Song, H. Y., Gao, X., Goeddel, D. V., Cao, Z., and Rothe, M. (1997) *Cell* **90,** 373–383
39. Seed, B., and Aruffo, A. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 3365–3369

40. Schreiber, E., Matthias, P., Müller, M. M., and Schaffner, W. (1989) *Nucleic Acids Res.* **17,** 6419
41. Force, W. R., Tillman, J. B., Sprung, C. N., and Spindler, S. R. (1994) *J. Biol. Chem.* **269,** 8863–8871
42. Smith, D. B., and Johnson, K. S. (1988) *Gene* (*Amstr.*) **67,** 31–40
43. Sun, S.-C., Elwood, J., and Greene, W. C. (1996) *Mol. Cell. Biol.* **16,** 1058–1065
44. Holbrook, N., Gulino, A., and Ruscetti, F. (1987) *Virology* **157,** 211–219
45. Boucher, L.-M., Marengere, L. E. M., Lu, Y., Thukral, S., and Mak, T. W. (1997) *Biochem. Biophys. Res. Commun.* **233,** 592–600
46. Lee, S. Y., Lee, S. Y., and Choi, Y. (1997) *J. Exp. Med.* **185,** 1275–1285
47. Regnier, C. H., Tomassetto, C., Moog-Lutz, C., Chenard, M.-P., Wendling, C., Basset, P., and Rio, M.-C. (1995) *J. Biol. Chem.* **270,** 25715–25721
48. Beg, A. A., and Baltimore, D. (1996) *Science* **274,** 782–784
49. Van Antwerp, D. J., Martin, S. J., Kafri, T., Green, D. R., and Verma, I. M. (1996) *Science* **274,** 787–789
50. Wang, C.-Y., Mayo, M. W., and Baldwin, J. A. S. (1996) *Science* **274,** 784–787