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TNF Receptor Family Member BCMA (B Cell Maturation) Associates with TNF Receptor-Associated Factor (TRAF) 1, TRAF2, and TRAF3 and Activates NF-κB, Elk-1, c-Jun N-Terminal Kinase, and p38 Mitogen-Activated Protein Kinase[1]

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BCMA (B cell maturation) is a nonglycosylated integral membrane type I protein that is preferentially expressed in mature B lymphocytes. Previously, we reported in a human malignant myeloma cell line that BCMA is not primarily present on the cell surface but lies in a perinuclear structure that partially overlaps the Golgi apparatus. We now show that in transiently or stably transfected cells, BCMA is located on the cell surface, as well as in a perinulear Golgi-like structure. We also show that overexpression of BCMA in 293 cells activates NF-κB, Elk-1, the c-Jun N-terminal kinase, and the p38 mitogen-activated protein kinase. Coimmunoprecipitation experiments performed in transfected cells showed that BCMA associates with TNFR-associated factor (TRAF) 1, TRAF2, and TRAF3 adaptor proteins. Analysis of deletion mutants of the intracytoplasmic tail of BCMA showed that the 25-aa protein segment, from position 119 to 143, conserved between mouse and human BCMA, is essential for its association with the TRAFs and the activation of NF-κB, Elk-1, and c-Jun N-terminal kinase. BCMA belongs structurally to the TNFR family. Its unique TNFR motif corresponds to a variant motif present in the fourth repeat of the TNFRI molecule. This study confirms that BCMA is a functional member of the TNFR superfamily. Furthermore, as BCMA is lacking a "death domain" and its overexpression activates NF-κB and c-Jun N-terminal kinase, we can reasonably hypothesize that upon binding of its corresponding ligand BCMA transduces signals for cell survival and proliferation. *The Journal of Immunology,* **2000, 165: 1322–1330.**

The TNF-related cytokines are a large family of pleiotropic mediators of host defense and immune system regulators. Those that are integral membrane proteins act locally through cell-to-cell contact, and those that are secreted proteins act on distant target cells. The TNFR are a heterologous family, of which 18 members are known. They mediate the action of TNF-related cytokines leading to cell death or cell proliferation and differentiation (1, 2). Most members of the TNFR family are type I transmembrane proteins with an extracellular ligand-binding domain, a single membrane-spanning region, and a cytoplasmic region that activates cell functions (3). The common characteristic of all TNFR family members is the repetition of a six-cysteine motif in the extracellular N-terminal part of the molecule. In contrast to the extracellular parts of the receptors, the sequences of the cytoplasmic tails are generally dissimilar, and none possess sequences suggestive of catalytic activity. However, several motifs in the C-terminal part of TNFR have been shown to bind protein factors transducing the signal initiated by ligand binding and receptor trimerization. One of these motifs, the "death domain," is present in TNFRI, Fas, DR3, DR4, and DR5 and is responsible for the capacity of these receptors to induce apoptosis (4, 5). A second group of motifs binds signal transducers, TNFR-associated factors (TRAFs).[4] TRAFs interact directly with several TNFRs, like TNFRII, CD40, CD30, and lymphotoxin β receptor (6–9) and with the EBV oncogene LMP1 (10). TRAF2, TRAF5, and TRAF6 mediate the activation of the transcriptional factor NF-κB (11–13) and activate the c-Jun N-terminal protein kinase (JNK) (14). TRAF6 also mediates the activation of extracellular signal-regulated kinase (ERK) (15).

Recently, we identified of a novel TNFR (16, 17) through the molecular analysis of a t(4;16) translocation (16, 18), characteristic of a malignant human T cell lymphoma. The gene product is selectively expressed in mature B lymphocytes (19) and was therefore named BCMA for B cell maturation protein. The BCMA gene codes for a nonglycosylated integral membrane type I protein. The N-terminal part of both mouse and human proteins contains a conserved six-cysteine motif (17). A sensitive method of sequence analysis, hydrophobic cluster analysis (20), indicated that this conserved motif is similar to the six-cysteine repeat motif found in the

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[4] Abbreviations used in this paper: TRAF, TNFR-associated factor; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-related kinase; BCMA, B cell maturation; HA, hemagglutinin; h, human; m, mouse; ATF, activating transcription factor; GFP, green fluorescence protein; MEK, MAPK/ERK kinase; MEKK, MEK kinase; PI3-K, phosphatidylinositol 3-kinase.

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extracellular part of TNFRs. There are two notable differences between the BCMA protein and other members of the TNFR family. The first is that BCMA contains only one six-cysteine-rich motif, whereas the members of the TNFR family contain more than one copy. The second is that the six-cysteine motif of BCMA is not the canonical motif of TNFRs but corresponds to a variant motif present in the fourth repeat of the TNFRI molecule. The full name for BCMA in the new TNF nomenclature scheme is TNFRSF17. The human BCMA gene is the first TNFR gene that has been implicated in chromosome translocation.

We report a study of the cellular localization of BCMA in transiently and stably transfected cells. We show that overexpression of BCMA activates the NF-κB, Elk-1, p38, and JNK. We also studied the association of the six known TRAFs with BCMA and defined the region of the BCMA protein responsible for this association.

Materials and Methods

Abs and reagents

The rabbit polyclonal anti-TRAF1 (H-132), anti-TRAF2 (C-20), anti-TRAF3 (H-122), anti-TRAF5 (H-257), anti-JNK1 (sc-474), and goat polyclonal anti-TRAF4 (N-16) and anti-goat HRP-conjugated Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). M2 anti-FLAG mAb, M2 mAb bound to agarose beads, and FLAG peptide and protease inhibitor mixture were purchased from Sigma-Aldrich (St. Louis, MO). Anti-F mAb was a generous gift from M. C. Rio (Institut National de la Santé et de la Recherche Médicale, Unité 184, Strasbourg, France). 12CA5 anti-hemagglutinin (anti-HA) mAb was purchased from Roche Diagnostics (Somerville, NJ). PE-, FITC-, and HRP-conjugated goat anti-mouse IgG polyclonal Abs and HRP-conjugated donkey anti-rabbit IgG polyclonal Abs were purchased from Immunotech (Marseille, France). Rabbit polyclonal anti-phosphatidylinositol 3 kinase (PI3-K) p85 Abs were obtained from Upstate Biotechnology (Lake Placid, NY). RPMI 1640, DMEM, FCS, additional reagents for cell culture, optimem, and lipofectamine were purchased from Life Technologies (Grand Island, NY).

Primers

The following primers were used in this study: BCMA5′ATG (5′-AAGCTTATGTTGCAGATGGCTGGGCA-3′), BCMA3′TAA (5′-GGATCCTTACCTAGCAGAAATTGATTTC-3′), 37 (5′-CCCAAGCTTATGGCTGGGCAGTGCTCC-3′), 43 (5′-CGCGGATCCTTATGGTTCAGAGCTTATCTTCCT-3′), AH7 (CGCGGATCCTTACCTAGCAGAAATTGATTTCT-3′), AH9 (5′-CCGCTCGAGGGCGCAACAGTGTTTCCACA-3′), AH10 (GGAAGATCTCTAACGACATCTAAAACACCAG-3′), BFL1 (5′-AACTGCAGCTGGGCAGTGCTCCCAAAA-3′), BFL2 (5′-CGGGATCCTTAATAGTCATTCGTTTTCGTGGTG-3′), BFL3 (5′-CGGGATCCTTAGCAATGGTCATAGTCGACCT-3′), BFL4 (5′-CGGGATCCTTAGCCTCTCGGAAGAATAATTTC-3′), and BFL5 (5′-CGGGATCCTTAGTTTTTAAACTCGTCCTTTAATG-3′). All primers used in this study were purchased from Genset (Paris, France).

Expression vectors

A full-length human BCMA (h184) was amplified by PCR from human cDNA using the BCMA5′ATG and BCMA3′TAA primers; a fragment encoding the N-terminal and transmembrane parts of hBCMA (h84) was amplified by PCR using primers 37 and 43. The PCR fragments were digested with *Bam*HI and *Hin*dIII restriction enzymes and ligated into the *Bam*HI and *Hin*dIII sites of the vector pcDNA3 (Invitrogen, Groningen, The Netherlands).

A full-length mouse BCMA was amplified by PCR from a mouse cDNA library with the primers AH9 and AH10. The PCR fragment was digested with *Xho*I and *Bgl*II and ligated into the *Xho*I and *Bgl*II sites of the vector pDEB (21), giving rise to a fusion encoding a N-terminal HA-tagged mouse BCMA (HAm185). The HA-tagged mBCMA was digested with *Eco*RI and *Not*I and ligated into the *Eco*RI and *Not*I sites of the vector pcDNA3.

N-terminal FLAG-tagged hBCMA deletion mutants were constructed by PCR amplification using the following pairs of primers: BFL1 and AH7 for FLAG-hBCMA without deletion (Fh184), BFL1 and BFL2 for FLAG-hBCMAΔ165–184 (Fh164), BFL1 and BFL3 for FLAG-hBCMAΔ144–184 (Fh143), BFL1 and BFL4 for FLAG-hBCMAΔ119–184 (Fh118), and BFL1 and BFL5 for FLAG-hBCMAΔ92–184 (Fh91). All PCR products were digested with *Pst*I and *Bam*HI and ligated between the *Pst*I and *Bam*HI sites of the vector pSG5-FLAG (22). All expression vectors were constructed by standard recombinant DNA procedures. The sequence of the plasmids constructed by PCR amplification were subsequently verified by dideoxy sequencing.

The vectors pSG5hTRAF1 (10), pSG5hTRAF2, pSG5FLAGhTRAF2 (23), pSG5FLAGhTRAF1, pSG5hTRAF3, pSG5FLAGhTRAF3 (24), pMEFLAGmTRAF5 (13), pMEFLAGmTRAF6 (25), pEBBhTRAF5 (26), pcLMP1 (27), pcDNA3TRAF2.DN (TRAF2Δ6–86) (23), pGEX-Jun(1–79), pcDNA3-HA-JNK (28), and the β-galactosidase expression vector (pGK-βgal), in which expression is driven by the phosphoglucokinase promoter (22), have been already described. pAT3FhTRAF4 encoding human FLAG-tagged TRAF4 was a generous gift from Dr. Catherine Regnier (Strasbourg, France).

Cell lines and transfections

Human embryonic kidney 293, 293T, and 293EBNA and simian kidney COS7 cells were maintained in high-glucose DMEM supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin and were grown at 37°C in 5% CO_2. The 293EBNA cell line was purchased from Invitrogen and maintained in culture according to the supplier's instructions. The BJAB cell line is an EBV-negative Burkitt lymphoma cell line (29) and was cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin and grown at 37°C in 5% CO_2. Adherent cells were seeded in six-well plates (5×10^5 cells per well) in 2 ml of complete medium, incubated at 37°C in 5% CO_2 for 20–24 h, and transfected with lipofectamine according to the manufacturer's instructions, using 1 μg of total plasmid DNA, for 6 h. BJAB cells were transfected by electroporation (960 μF, 210 V) in 400 μl optimem medium using a Bio-Rad Gene Pulser apparatus (Bio-Rad, Richmond, CA). Cell extracts were tested for gene expression 24–48 h after transfection. To establish cells stably expressing BCMA, 293 cells were transfected with HAm185-expressing vector and were selected in high-glucose DMEM, 10% FCS, in presence of 400 μg/ml geneticin. Geneticin-resistant clones were screened by immunoblotting for BCMA expression.

Luciferase reporter system for NF-κB, Elk-1, and JNK

The NF-κB, Elk-1, and JNK activation assays were performed using the corresponding luciferase reporter PathDetect Reporting systems purchased from Stratagene (La Jolla, CA).

Luciferase and β-galactosidase assays

Transfected cells were washed twice with PBS and lysed with reporter lysis buffer (Promega, Madison, WI). The luciferase activity was measured using the reporter assay system (Promega). β-galactosidase activity was measured using the luminescent β-galactosidase reporter system (Clontech, Palo Alto, CA) in a Packard luminometer analyzer (Packard, Meriden, CT). Measurements of luciferase were normalized to β-galactosidase activity and are expressed as a ratio to values obtained from cells treated with vector alone. The relative luciferase activities given are representative of triplicate assays in three independent experiments.

Determination of JNK activity

JNK activity was determined as described previously (28) with minor modifications. Transfected cells were lysed in 10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% Nonidet P-40, 0.5 mM sodium vanadate, 0.2 mM PMSF, and 10% glycerol. Lysates were clarified by centrifugation, and HA-tagged JNK was immunoprecipitated using anti-HA mAb 12CA5. Immune complexes were collected on protein-G Agarose beads, washed three times in lysis buffer, once in kinase reaction buffer (12.5 mM MOPS, pH 7.5, 12.5 mM β-glycerophoshate, 7.5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM NaF, 0.5 mM sodium vanadate), and resuspended in 30 μl of the same buffer containing 2 μg of GST-Jun, 20 μM unlabeled ATP, and 5 μCi [γ-^{32}P]ATP. After incubation at 20°C for 30 min, kinase reaction products were analyzed by SDS-PAGE and autoradiography. Part of the immunoprecipitated material was resolved by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and immunoblotted with anti-JNK polyclonal Abs to check that the same amount of HA-JNK was used in each case.

Determination of p38 and ERK activity

The activity of these two kinases was assayed using the corresponding assay kit purchased from New England Biolabs (Beverly, MA). Briefly, transfected cells were lyzed, and the active phosphorylated kinase was immunoprecipitated using specific mAbs. The immunoprecipitated protein was assayed for its ability to phosphorylate activating transcription factor

(ATF) 2 (p38) or Elk-1 (ERK) substrates. Analysis of phosphorylated substrates was performed by Western blotting using specific polyclonal phosphoantibodies.

Coimmunoprecipitation experiments

COS7 cells were cotransfected with one vector encoding a TRAF and one vector encoding one of the various FLAG-tagged BCMA constructs. Eighteen to 24 h after transfection, cells were lysed in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1.5 mM EDTA, 10% glycerol, 0.1% Nonidet P-40, and a protease inhibitor mixture) by incubation for 1 h at 4°C, and the supernatant was then clarified by centrifugation. One-fortieth of this lysate (input) was conserved to test the efficiency of transfection, and the rest was incubated for 2 h, at 4°C, with M2 monoclonal anti-FLAG Ab, covalently bound to agarose beads. The beads were washed three times with the lysis buffer, and the bound proteins were eluted twice by addition of 250 μM FLAG peptide diluted in PBS. The eluate and the input were analyzed by PAGE and transferred onto a polyvinylidene difluoride membrane (Hybond-P; Amersham, Little Chalfont, U.K.). The presence of BCMA and of the various TRAFs was tested by immunoblotting using M2 Ab to evidence FLAG-tagged BCMA constructs and the corresponding anti-TRAF Ab for each TRAF. HRP-conjugated anti-rabbit, anti-goat, and anti-mouse IgG and SuperSignal Chemiluminescent substrate (Pierce, Rockford, IL) were used to reveal the blots.

Immunofluorescence staining and FACS analysis

For immunofluorescence observation, transfected ells were stained with M2 mAb then incubated with fluorescein-conjugated goat anti-mouse Ab before analysis under a Leica DM microscope (Leica, Deerfield, IL) as previously described (30). For FACS analysis, $\sim 5 \times 10^5$ cells per condition were stained with saturating concentrations of Ab, then incubated with PE-conjugated goat anti-mouse Ab before analysis in a FACScan flow cytometer (Becton Dickinson, San Diego, CA), as previously described (31). A minimum of 10,000 events per sample was analyzed. CellQuest software (Becton Dickinson) was used for data analysis.

Results

BCMA is present on both the surface of cells and in an intracellular perinuclear structure

In a previous study of BCMA localization, in the human myeloma cell line U266, we found most BCMA in a perinuclear Golgi-like structure (30). We further analyzed the localization of BCMA by transfection experiments in two cell lines: the human B lymphocyte BJAB cell line and the monkey kidney COS7 cell line. BJAB cell line has been chosen because it expresses detectable amount of BCMA mRNA, has nondetectable amounts of BCMA protein (data not shown), and can be transiently transfected with high efficiency (50–60%). On the contrary, COS7 cell line does not express BCMA. The cell lines were transfected with two vectors, one coding for a FLAG-tagged full-length hBCMA (Fh184) and a second one coding for a FLAG-tagged hBCMA construct lacking the entire intracellular cytoplasmic tail (Fh91), together with a green fluorescence protein (GFP) expression vector. Eighteen hours after transfection, cells were stained with M2 anti-FLAG Ab and a secondary PE-conjugated anti-mouse IgG. The GFP-expressing cell population was gated, and the presence of FLAG-tagged proteins on the surface and intracellularly was determined by two-color cytofluorometry (Fig. 1*A*). Full-length hBCMA and the mutant hBCMA missing its cytoplasmic tail were similarly distributed in both BJAB and COS7 cell lines. Both proteins displayed an intracytoplasmic localization, but were also present on the cell surface. The localization of full-length hBCMA was further examined by fluorescence microscopy. Our results (Fig. 1*B*) confirmed that BCMA was present on the cell surface. As previously observed in the myeloma U266 cell line, intracellular BCMA was detected in a perinuclear Golgi-like structure in both transfected BJAB and COS7 cell lines.

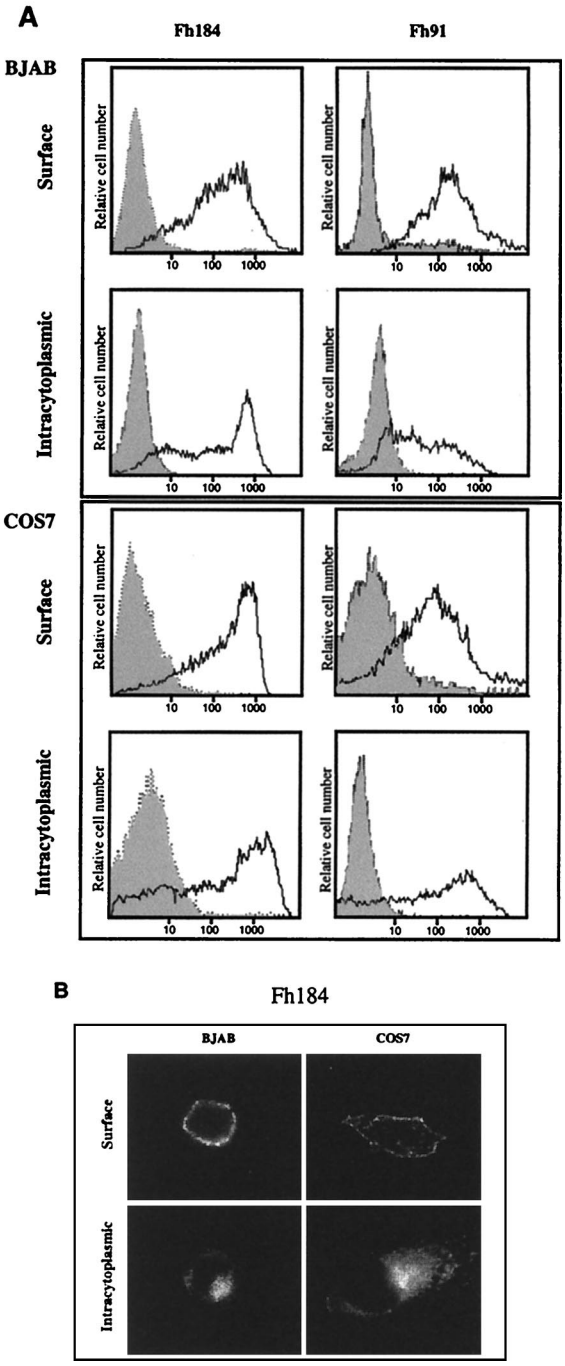

FIGURE 1. Transient expression of hBCMA in transfected BJAB and COS7 cell lines. The cell lines were transfected with two vectors, one coding for a N-terminal FLAG-tagged full-length hBCMA (Fh184) and a second one coding for a N-terminal FLAG-tagged hBCMA construct lacking the entire intracellular cytoplasmic tail (Fh91), together with a GFP expression vector. Eighteen hours after transfection, the cells were stained with the M2 anti-FLAG mAb and a secondary PE-conjugated anti-mouse IgG Ab, in the absence (for surface localization) and the presence (for intracellular localization) of permeabilizing detergent and analyzed by two-color flow cytometry (*A*). The GFP-expressing cell population was gated and further analyzed for FLAG-tagged protein expression (Fig. 1*A*). The intracellular and surface localization of Fh184 in BJAB and COS7 cells was also analyzed by fluorescence microscopy (*B*).

The cell localization of BCMA was also examined in stably transfected cells. To this end, 293 cell lines stably expressing HA-tagged mBCMA (HAm185) were derived and tested by flow cytometry for surface and intracytoplasmic expression of HAm185. The results obtained for one clone (clone 12) are shown in Fig. 2 and indicate both surface and intracytoplasmic localization of

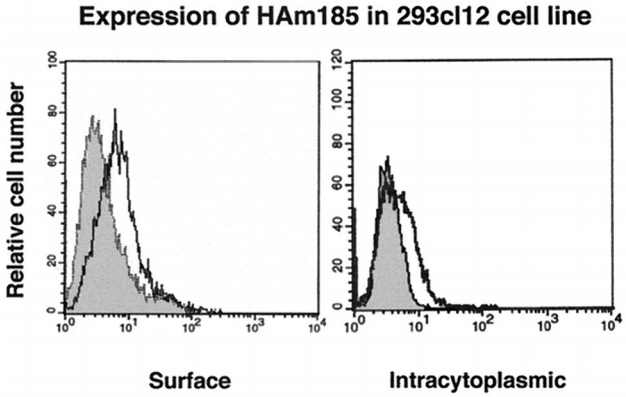

FIGURE 2. Stable expression of mBCMA in transfected 293 cells. The 293 cell line was transfected with HA-tagged mBCMA pcDNA3 expressing vector (HAm185). The cells were selected with 400 μg/ml of geneticin, and seven clones were isolated. The expression of HAm185 protein was tested in these clones by immunoblotting using the 12CA5 anti-HA mAb. Nontransfected 293 cells were used as negative control. The 293cl12 cells were stained with the 12CA5 anti-HA mAb and a secondary PE-conjugated anti-mouse IgG Ab, in the absence (for surface localization) and the presence (for intracellular localization) of permeabilizing detergent and analyzed by flow cytometry.

HAm185. Similar results have been obtained in two other clones tested (data not shown).

BCMA-mediated NF-κB activation

Most TNFRs, when overexpressed, activate NF-κB. To determine whether BCMA overexpression also results in NF-κB activation, 293 cells were cotransfected with CMV promoter-driven BCMA expression vectors together with a NF-κB luciferase reporter plasmid. Overexpression of hBCMA (h184) induced a 12-fold activation of NF-κB over the activation level obtained using the empty vector (Fig. 3*A*). Similarly, overexpression of mBCMA (HAm185) gave rise to a 10-fold activation of NF-κB. These activation levels were in the range of that observed (12-fold) using LMP1, a known activator of this nuclear factor. A dose-response curve was plotted and showed that 100 ng of hBCMA or mBCMA expression vectors were sufficient for maximal NF-κB activation (data not shown). As expected, transfection of 293 cells with the deletion mutant of hBCMA, lacking the intracytoplasmic tail of the molecule, (h84), failed to activate NF-κB, confirming that the cytoplasmic tail of BCMA is essential for transducing a signal and activating NF-κB.

To determine which sequence within BCMA cytoplasmic tail is necessary for NF-κB induction, we constructed a series of SV40 promoter-driven vectors encoding N-terminal FLAG-tagged deletion mutants of hBCMA and tested these mutants for NF-κB activation (Fig. 3*C*) in 293T cells. The deletion mutants Fh164 and Fh143, lacking the C-terminal 20 and 41 aa, respectively, had the same NF-κB activation capacity as the full-length BCMA molecule, Fh184. In contrast, mutants Fh118 and Fh91 did not activate NF-κB. The level of expression of the deletion mutant proteins was tested by immunoblotting and was found to be approximately similar (Fig. 3*D*). Therefore, the protein segment between amino acid residues 119 and 143 of BCMA is necessary for the activation of NF-κB. Interestingly, this sequence is highly conserved in hBCMA and mBCMA (Fig. 3*B*).

BCMA activates the mitogen-activated protein kinase (MAPK) pathway

We next examined the activation of the nuclear factor Elk-1 using a luciferase reporter system. Elk-1 is a substrate for the MAPKs:

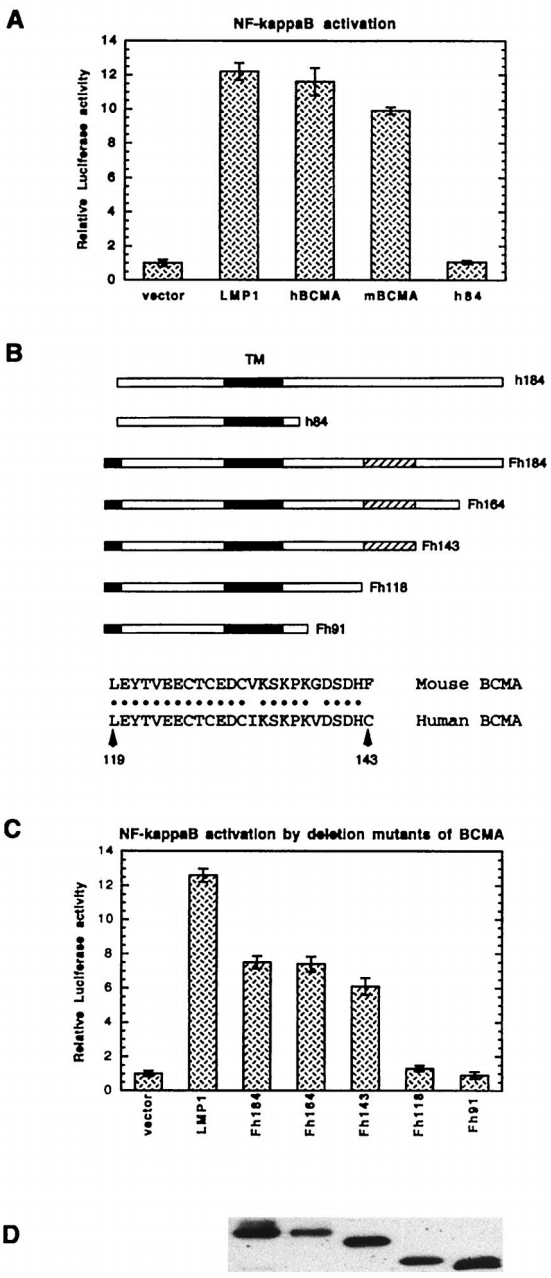

FIGURE 3. BCMA overexpression induces NF-κB activation. *A*, 293 cells were cotransfected with luciferase reporter plasmid and 100 ng of one of pcDNA3 (vector), pcDNA3LMP1 (LMP1), pcDNA3-hBCMA (full-length hBCMA), pcDNA3-HA tagged mBCMA185 (full-length mBCMA), and pcDNA3-hBCMA84(h84), which contains only the extracellular and transmembrane regions of hBCMA. *B*, Truncations of hBCMA cytoplasmic tail were obtained by standard PCR in pSG5FLAG. A schematic representation of these mutants is shown. Black blocks denote the transmembrane region (TM), gray blocks denote FLAG tags, and the hatched part represents the amino acid residue region between positions 119 and 143 essential for the activation of NF-κB. The sequences of human and mouse BCMA proteins in this region are shown to illustrate the similarity. *C*, 293T cells were cotransfected with luciferase reporter plasmid and 100 ng of one of empty vector (F), Fh184, Fh164, Fh143, Fh118, and Fh91. NF-κB activation was measured as described in *Materials and Methods*. The pGK-β-galactosidase plasmid encoding β-galactosidase was cotransfected in every sample to normalize transfection efficiencies. Forty-eight hours after transfection, cells were harvested, lysed, and analyzed for luciferase and β-galactosidase activity. Luciferase values were normalized to β-galactosidase activity, and the results are displayed as a multiple of the induction by vector alone. A representative result of three independent experiments is shown. Error bars denote SDs for triplicate samples. *D*, The level of production of deletion mutant proteins in 293T cells was tested by Western blot using the M2 anti-FLAG mAb.

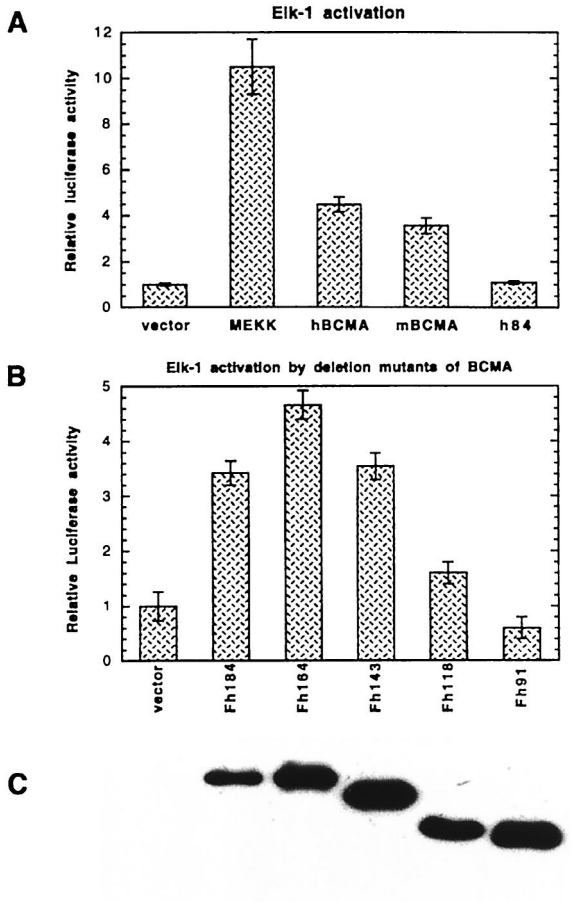

FIGURE 4. BCMA overexpression activates Elk-1 nuclear factor. *A*, 293 cells were cotransfected with the corresponding Pathfinder reporter system plasmids and 100 ng of empty vector (vector), LMP1, full-length hBCMA, HA-tagged full-length mBCMA, or h84, which contains only the extracellular/transmembrane region of hBCMA. MEK1-encoding plasmid was used as a positive control. *B*, 293T cells were cotransfected with the corresponding Pathfinder reporter system plasmids and 100 ng of empty pSG5FLAG (vector), Fh184, Fh164, Fh143, Fh118, or Fh91. Elk-1 activation was measured as described in *Materials and Methods*. The pGK-β-galactosidase plasmid encoding β-galactosidase was cotransfected in every sample to normalize transfection efficiencies. Twenty-four (for 293 cells) and 48 h (for 293T cells) after transfection, cells were harvested, lysed, and analyzed for luciferase and β-galactosidase activities. Luciferase values were normalized to β-galactosidase activity, and the results are displayed as a multiple of the induction by vector alone. A representative result of three independent experiments is shown. Error bars denote SDs for triplicate samples. *C*, The level of expression of deletion mutant proteins in 293T cells was tested by Western blot using the M2 anti-FLAG mAb.

JNK, p38, and ERK. Overexpression of hBCMA in 293 cells activated Elk-1 to a level 4.5-fold higher than that obtained using the empty vector (Fig. 4*A*). Similar results were obtained using mBCMA (3.5-fold activation). As expected, the mutant hBCMA h84 failed to activate Elk-1. MAPK/ERK kinase (MEK) 1 overexpression was used as a positive control for Elk-1 activation (10-fold). The activation of Elk-1 by the different deletion mutants of BCMA was also studied in 293T cells (Fig. 4*B*). Fh184, Fh164, and Fh143 constructions activated Elk-1 3.5-, 5-, and 2-fold, respectively, whereas Fh118 and Fh91 mutants gave no activation of this nuclear factor. The level of expression of the deletion mutant proteins was tested by immunoblotting and was found similar (Fig. 4*C*). Therefore the protein segment between amino acid residues 119

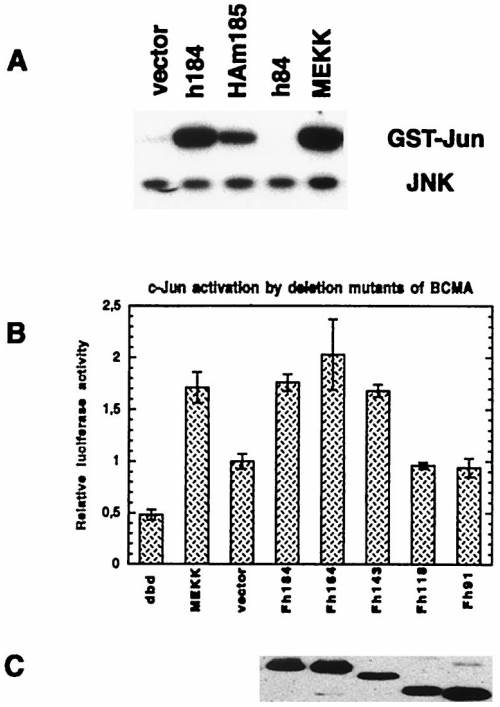

FIGURE 5. Overexpression of BCMA activates JNK. *A*, 293 cells were cotransfected with 100 ng of HA-JNK plasmid and 100 ng of empty vector (vector), pFRMEKK vector from Stratagene's Pathfinder reporter system (MEKK), full-length hBCMA, HA-tagged full-length mBCMA, or h84, which contains only the extracellular/transmembrane region of human BCMA. The immunoprecipitated HA-JNK was used in in vitro phosphorylation experiments of GST-Jun substrate, and the radioactively phosphorylated GST-Jun was analyzed by SDS-PAGE and autoradiography. Blotting with anti-JNK polyclonal Abs confirmed that the same amount of JNK was present in each of the phosphorylation mixtures. *B*, 293EBNA cells were cotransfected with the corresponding Pathfinder reporter system plasmids and 100 ng of empty pSG5FLAG (vector), Fh184, Fh164, Fh143, Fh118, or Fh91. c-Jun-dependent luciferase activity was measured 48 h after transfection. The MEKK plasmid was used as a positive control for JNK activation. Vector encoding β-galactosidase was cotransfected in every sample to normalize transfection efficiencies. Data are shown as the mean ± SD of triplicate samples and represent one of the four independent experiments, all of which gave similar results. *C*, The level of expression of deletion mutant proteins in 293EBNA cells was tested by Western blot using the M2 anti-FLAG mAb.

and 143 of BCMA is essential for the activation of the nuclear factor Elk-1.

To assess the ability of BCMA to activate JNK, we transiently cotransfected 293 cells with an HA-tagged JNK vector together with one of pcDNA vectors expressing h184, HAm185, h84, pcDNA3, and pFRMEKK vectors. The activation of JNK was examined by measuring phosphorylation of its substrate, GST-Jun. Overexpression of MEK kinase (MEKK) was used as a positive control. The overexpression of either human or mouse BCMA significantly increased the amount of phosphorylated GST-Jun, as compared with that of the cells transfected with the empty vector or with the h84 mutant of hBCMA, the mutant lacking the cytoplasmic tail (Fig. 5*A*). These data indicate that overexpression of BCMA activates JNK. We also tested the level of activation of c-Jun by the SV40 promoter-driven vectors expressing deletion mutants of BCMA using a luciferase reporter system. As the background level was high in 293T cells, we have used 293EBNA cells in which we have obtained a lower background. The expression of Fh184, Fh164, and Fh143 mutants resulted in a 2-fold activation of

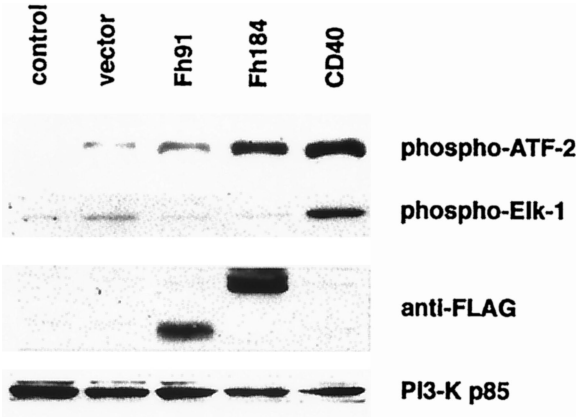

FIGURE 6. Overexpression of BCMA activates the p38 MAPK and does not activate the ERK one. Two sets of 293T cells were transfected with no plasmid (control) and 1 μg of each empty pSG5FLAG vector (vector), Fh91, Fh184, and a vector expressing full-length human CD40 (CD40). Twenty-four hours after transfection, the cells were lysed and 300 μg of lysate were assayed for p38 (phospho-ATF-2) and ERK (phospho-Elk-1) activity, using a pull-down dual assay kit. Two hundred micrograms of cell lysate was immunoprecipitated using M2 anti-FLAG mAb covalently bound to beads and assayed by Western blot using the M2 anti-FLAG mAb for the expression of Fh184 and Fh91 proteins. To verify the similar level of expression of proteins in 293T cells, 10 μg of cell lysate were assayed by Western blot using a rabbit polyclonal anti-PI3-Kp85 Ab.

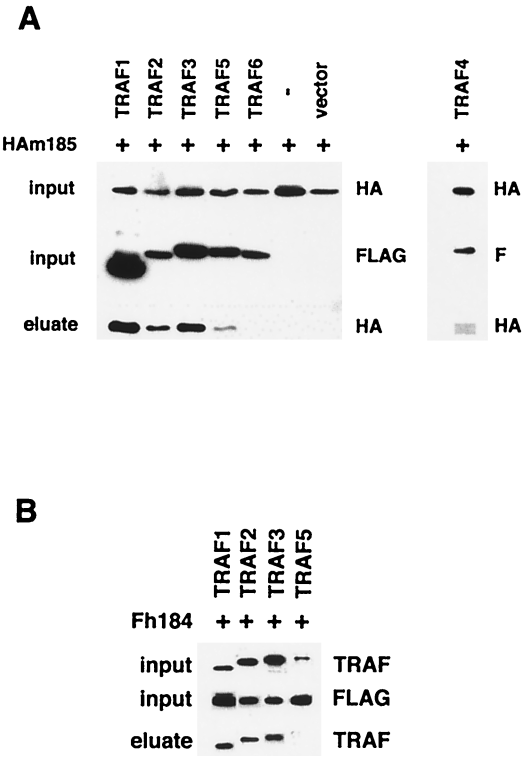

FIGURE 7. TRAF1, TRAF2, and TRAF3 coimmunoprecipitate with BCMA. *A*, COS7 cells were cotransfected with HA-tagged full-length mBCMA-HAm185 and plasmids encoding human FLAG-tagged TRAF1, TRAF2, TRAF3, F-tagged human TRAF4, or FLAG-tagged mouse TRAF5 or TRAF6. Cells were lysed, and the lysate was immunoprecipitated with M2 anti-FLAG mAb, except for TRAF4, which was immunoprecipitated with anti-F mAb. After washing, the bound proteins were eluted by addition of FLAG peptide (TRAF4 eluted directly by addition of gel loading buffer), and the eluate was electrophoresed, blotted onto a membrane, and tested for the presence of coimmunoprecipitated TRAFs. One-fortieth of the lysate (input) was electrophoresed, blotted, and tested for the expression of the various TRAFs using anti-FLAG (TRAF1, TRAF2, TRAF3, TRAF5, TRAF6), anti-F (TRAF4), and anti-HA (mBCMA) Abs. The eluate of each immunoprecipitation was tested for the presence of associated HA-tagged mouse BCMA. *B*, COS7 cells were cotransfected with Fh184 and plasmids encoding human TRAF1, TRAF2, TRAF3, or TRAF5. Cells were lysed, and the lysate was immunoprecipitated with M2 anti-FLAG mAb. After washing, the bound proteins were eluted by addition of FLAG peptide, and the eluate was electrophoresed, blotted onto a membrane, and tested for the presence of coimmunoprecipitated TRAFs. One-fortieth of the lysate (input) was tested for the expression of the various TRAFs and of the Fh184 (*B*). The eluate of each immunoprecipitation was tested for the presence of corresponding TRAFs.

c-Jun phosphorylation, whereas the Fh119 and Fh91 mutants gave lower induction levels than the empty vector (Fig. 5*B*). The level of expression of the deletion mutant proteins was tested by Western immunoblotting and was found similar (Fig. 5*C*).

The ability of BCMA to activate p38 and ERK MAPKs were assayed using a nonradioactive pull-down MAPK assay kit. 293T cells were transiently transfected with no plasmid (negative control) and with 1 μg of each pSG5FLAG empty vector, Fh91, Fh184, and a human CD40-expressing vector (positive control). The level of activation of the kinases was tested by measuring the phosphorylation of ATF-2 (for p38) or Elk-1 (for ERK). The results obtained are shown in Fig. 6. The overexpression of Fh184 significantly increased the amount of phosphorylated ATF-2, as compared with that of the cells transfected with no or empty vector or with the Fh91 mutant of hBCMA, which is lacking the cytoplasmic tail. On the contrary, the overexpression of Fh184 did not increase the amount of phosphorylated Elk-1. These data indicate that overexpression of BCMA activates the p38 kinase and not the ERK one. Cell lysates were immunoprecipitated for FLAG-tagged proteins and assayed by Western blot using M2 anti-FLAG mAb; the results showed that similar amounts of Fh91 and Fh184 have been produced during transient transfection of the cells. Finally, the amount of protein used for the experiment has been assessed by Western blot using a rabbit polyclonal anti-PI3-Kp85 Ab and found to be similar.

Overexpression of BCMA activates the NF-κB and Elk-1 nuclear factors and the JNK and p38 MAPKs; furthermore, the cytoplasmic protein segment comprised between positions 119 and 143 is essential for NF-κB, Elk-1, and JNK activation and is highly conserved in hBCMA and mBCMA.

Functional and biochemical mapping of the BCMA intracytoplasmic tail

We studied the association of the six known TRAFs with BCMA. COS7 cells were cotransfected with the mouse HAm185 plasmid and one of the plasmids encoding FLAG-tagged human TRAF1, TRAF2, TRAF3, F-tagged human TRAF4, or FLAG-tagged mouse TRAF5 or TRAF6. The cells were lysed 48 h later, and proteins were immunoprecipitated with the M2 anti-FLAG mAb for TRAF1, TRAF2, TRAF3, TRAF5, or TRAF6 or with anti-F mAb for TRAF4. Coimmunoprecipitated HAm185 was detected by immunoblotting with anti-HA mAb (Fig. 7*A*). The mBCMA associates strongly with TRAF1, TRAF2, and TRAF3 molecules, weakly with TRAF5, and not with TRAF4 and TRAF6. To validate these results, a second series of experiments was performed: COS7 cells were cotransfected with the Fh184 plasmid and an expression plasmid for either human TRAF1, TRAF2, TRAF3, or TRAF5. The transfected cells were lysed 24 h later, and FLAG-tagged hBCMA was immunoprecipitated with M2 anti-FLAG mAb. Coimmunoprecipitated TRAFs were detected by immunoblotting with corresponding anti-TRAF Ab. The hBCMA, under

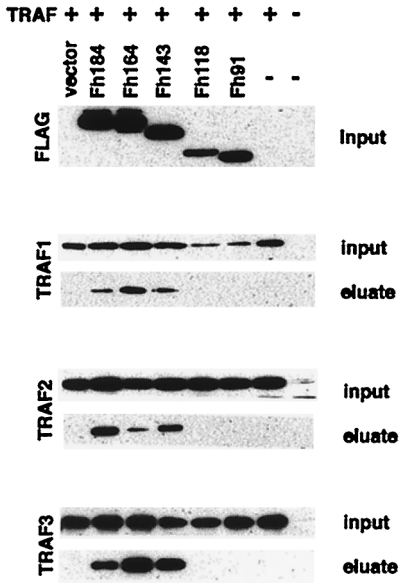

FIGURE 8. The 25-aa protein segment (119–143) of hBCMA is required for the association with TRAF1, TRAF2, and TRAF3. COS7 cells were cotransfected with one of the plasmids encoding human TRAF1, TRAF2, and TRAF3 and with one of empty pSG5FLAG (vector), Fh184, Fh164, Fh143, Fh118, and Fh91 plasmids. Cells were lysed, and the lysate was immunoprecipitated with M2 anti-FLAG mAb. After washing, the bound proteins were eluted by addition of FLAG peptide, and the eluate was electrophoresed, blotted onto a membrane, and tested for the presence of coimmunoprecipitated TRAFs. One-fortieth of the lysate (input) was tested for the expression of the various TRAFs and the deletion mutants of FLAG-tagged hBCMA. The input of FLAG-tagged mutants shown corresponds to the coimmunoprecipitation experiments with TRAF1. The coexpression of the two other TRAFs (TRAF2 and TRAF3) gave similar results.

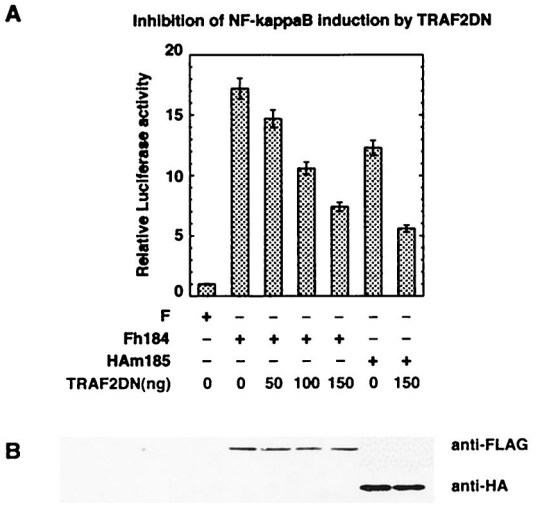

FIGURE 9. Dominant negative TRAF2 protein inhibits BCMA-mediated NF-κB activation. *A*, 293T cells were cotransfected with luciferase reporter plasmid, 100 ng of one of empty vector (F), Fh184, HAm185, and increasing amounts of a pcDNA3TRAF2DN expressing vector (50, 100, and 150 ng). NF-κB activation was measured as described in *Materials and Methods*. The pGK-β-galactosidase plasmid encoding β-galactosidase was cotransfected in every sample to normalize transfection efficiencies. Forty-eight hours after transfection, cells were harvested, lysed, and analyzed for luciferase and β-galactosidase activity. Luciferase values were normalized to β-galactosidase activity, and the results are displayed as a multiple of the induction by vector alone. A representative result of three independent experiments is shown. Error bars denote SDs for triplicate samples. *B*, The level of expression of Fh184 and HAm185 was assessed by Western blot using the M2 anti-FLAG Ab for Fh184 and the 12CA5 anti-HA Ab for HAm185.

the experimental conditions used, associated only with TRAF1, TRAF2, and TRAF3 (Fig. 7*B*).

To identify sequences necessary for the association between BCMA and the various TRAF proteins, we studied the association of the different FLAG-tagged mutants of hBCMA with human TRAF1, TRAF2, and TRAF3. COS7 cells were cotransfected with one of the Fh184, Fh164, Fh143, Fh118, or Fh91 expression vectors and one of the TRAF1, TRAF2, or TRAF3 vectors. The Fh184, Fh164, and Fh143 constructions associated with the TRAF1, TRAF2, and TRAF3, whereas the Fh118 and Fh91 deletion mutants did not bind any of the three TRAFs tested (Fig. 8).

The data presented indicate that the BCMA activates NF-κB, Elk-1, and JNK and associates with TRAF1, TRAF2, and TRAF3. The protein segment between the amino acid sequence positions 119 and 143 in the cytoplasmic tail of BCMA is required for both TRAF association and NF-κB, Elk-1, and JNK activation, consistent the TRAFs being involved in these activations.

A dominant negative form of TRAF2 decreases BCMA-mediated NF-κB activation

The requirement of TRAF2 for BCMA-mediated NF-κB activation was tested using a vector that encodes the TRAF2 dominant-negative mutant TRAF2.DN(Δ6–86). This mutant, lacking the N-terminal RING finger domain, suppresses signaling of NF-κB by interacting with the receptor and preventing activation of specific endogenous TRAF2 molecules (11, 23, 32). Coexpression of Fh184 and HAm185 expression vectors with increasing amounts of TRAF2.DN expression vector, in transfected 293T cells, resulted in a dose-dependent inhibition of NF-κB activation (Fig. 9*A*). The highest concentration of added TRAF2.DN expression vector (150 ng) resulted in >50% inhibition of NF-κB activation for both hBCMA and mBCMA. The level of expression of either Fh184 or HAm185 proteins was tested by immunoblotting and was found unmodified until the addition of 150 ng of TRAF2.DN-expressing vector (Fig. 9*B*). We cannot answer the question whether 100% inhibition of NF-κB activation can be obtained, because addition of higher amounts of TRAF2.DN vector resulted in a decrease of expression of Fh184 and HAm185 proteins.

Discussion

We addressed the issues of the localization of the BCMA protein and its signal transduction. In a previous study, we have characterized the BCMA gene and protein both in the human and the mouse. We showed that, in the human myeloma U266 cell line, the BCMA protein is mainly found in a Golgi-like perinuclear structure (30). Functional TNFR members are localized at the cell surface, and therefore BCMA might also be found at the same location. Indeed, production of FLAG-tagged BCMA in BJAB and COS7 cell lines allowed us to demonstrate the presence of BCMA on the cell surface, as well in a perinuclear Golgi-like structure. To test whether the intracytoplasmic tail of BCMA is responsible for the Golgi retention, as it has been reported for some proteins, we have also studied the localization of the mutant BCMA construct with its C terminal truncated. There were no differences in localization of the full-length and mutant BCMA proteins. To answer the question whether or not the surface localization of BCMA is the result of its transient overexpression, we have established 293 clones stably expressing BCMA. We have found that these clones have both a surface and intracytoplasmic localization BCMA. The Golgi-like localization of a TNFR protein has been described, in

human endothelial cells, in which most TNFR1 is Golgi-associated protein and little is found on the plasma membrane (33). Transfection experiments in human monocyte U937 and human endothelial ECV304 cell lines confirmed the Golgi localization of TNFR1 (34). Furthermore, it has been reported that, in human vascular smooth muscle cells, p53 activation transiently increased surface Fas (CD95) expression by transporting the protein from the Golgi complex to the plasma membrane (35). Therefore, it is possible that there is a mechanism modulating BCMA expression on the cell surface of normal B lymphocytes by controlling its transport from a Golgi-like structure.

Members of the TNFR superfamily associate either directly or indirectly with TRAFs that recruit and activate downstream signal transducers. TRAFs are adaptor proteins that further propagate the signal elicited by TNF, which causes an activation of nuclear factors, namely the NF-κB, Elk-1, and JNK. We investigated whether BCMA overexpression falls into the same signal propagation scheme. Our results can be interpreted as follows.

The overexpression of BCMA activates the MAPK pathway, especially JNK and p38 kinase, and the nuclear factors NF-κB and Elk-1. As expected, a mutant BCMA lacking the cytoplasmic tail failed to activate any of the factors studied. In this respect, BCMA follows the scheme of other members of the TNFR family. Analysis of the activation of JNK, NF-κB, and Elk-1 by deletion mutants of BCMA indicated that the same protein segment of 25 aa residues (119–143) is indispensable for the activation of these three proteins.

Coexpression of the different TRAF and BCMA evidenced association of TRAF1, TRAF2, and TRAF3 adaptor proteins with BCMA. Note that a faint association of mouse TRAF5 to mouse BCMA was observed; this result was not confirmed when we tested the association of either human TRAF5 with hBCMA or of mouse TRAF5 with hBCMA. We further showed that the protein segment (amino acid positions 119–143), which is essential for the activation of JNK, NF-κB and Elk-1, was also necessary for the association with TRAF1, TRAF2, and TRAF3, suggesting that the activation is achieved through the association of TRAF proteins. We have also showed that a dominant negative form of TRAF2 decreases the NF-κB activation mediated by BCMA overexpression.

Several TRAF binding motifs such as PXQXT/S (10), EXGKE (8), or VXX(T/S)XEE (36) have been identified in other TNFR members as associating with TRAF1, TRAF2, TRAF3, and TRAF5. None of these motifs is present in BCMA. However, major (P/S/A/T)X(Q/E)E and minor PXQXXD TRAF2-binding consensus sequences have recently been proposed (37). The major sequence motif is present in the protein segment (amino acid positions 119–143) of BCMA essential for both association of TRAFs and activation of JNK, NF-κB, and Elk-1, positions 122–125 (T^{122} V^{123} E^{124} E^{125}). Therefore, we are trying actually to verify whether this sequence motif is also necessary for the association of TRAF1 and TRAF3 with BCMA.

This study confirms that BCMA is a functional member of the TNFR superfamily. Furthermore, as BCMA is lacking a "death domain" and its overexpression activates NF-κB, p38, and JNK, we can reasonably hypothesize that upon binding of its corresponding ligand, BCMA transduces signals for cell survival and proliferation.

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